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## DETERMINATION OF DOPA OXIDASE ACTIVITY IN PIGMENTED TISSUES

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The term "activity of DOPA oxidase" is used in this paper to signify the enzymic oxidation of *l*-3,4-dihydroxyphenylalanine (DOPA) to pigmented products by the action of mammalian tissues, such as the Harding-Passey mouse melanoma<sup>1, 2</sup> and human melanotic melanoma.<sup>1</sup> We are aware that DOPA oxidase in mammalian tissues is regarded by some<sup>1, 2</sup> as distinct from tyrosinase, whereas other workers think that it is identical with the latter enzyme.<sup>4</sup> Methods based on the development of color from added DOPA have been used to measure the activity of DOPA oxidase in the ascomycete *Glomerella cingulata*,<sup>5</sup> and in *Neurospora*.<sup>3</sup> On the other hand, with darkly pigmented preparations of enzyme, the range of the electrophotometer may be exceeded, and the greater dilution that is required may render the enzyme ineffective.<sup>8</sup> The study described in this paper was designed for the development of a colorimetric method that is suitable for the determination of activity of DOPA oxidase in pigmented tissues.

### REAGENTS

*DOPA stock (1 mg. per ml.)*. Dissolve 0.1000 Gm. of *l*-3,4-dihydroxyphenylalanine (Mann Research Laboratories) in water and dilute to 100 ml. Store in the dark at  $-15^{\circ}\text{C}$ . and discard the solution if any color develops.

*Barbiturate buffer (0.1 M)*. Dissolve 20.6 Gm. of sodium diethylbarbiturate (Merck) in distilled water and dilute to 1000 ml.

### PROCEDURE

1. Fragment the tissue, or a weighed aliquot, in a Potter homogenizer with a small quantity of water and silica sand No. 140.
2. Dilute the suspension so that the concentration of tissue is 1:30, with DOPA being added during the dilution to a final concentration of 200  $\mu\text{g}$ . per ml. Remove 1 ml. for the assay of initial DOPA, and then immediately add an equal volume of acetone in order to stop the enzymic oxidation and precipitate the particulate matter, including melanin.<sup>6</sup>
3. Incubate the mixture for 1 hr. in a water-bath at  $37^{\circ}\text{C}$ ., with continuous aeration by means of a fine stream of air introduced through a capillary pipet. In order to avoid excessive evaporation, the air should be saturated with water vapor.

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4. Remove a second aliquot of 1 ml. for the assay of DOPA remaining after oxidation by the tissue, and immediately add an equal volume of acetone. Centrifuge both aliquots for 10 min. in an International No. 2 centrifuge at 2000 r.p.m., and pipet 1 ml. of the relatively clear supernatant fluids (containing a maximum of 100  $\mu$ g. of DOPA) into 10-ml. calibrated Klett tubes. Make up the volumes to 5 ml. with barbiturate buffer.

5. Measure the absorbencies ( $A_1$ ) of the 2 tubes in a Klett-Summerson photoelectric colorimeter at a wavelength of 420  $m\mu$ .

6. Incubate both tubes at 65 C. for 30 min., in order to permit the development of color from the auto-oxidation of DOPA.

7. Again read the absorbencies ( $A_2$ ) for the 2 tubes and calculate, by means of subtracting  $A_1$  from  $A_2$ , the density of color that results from DOPA.

8. Referring to a standard curve (Fig. 1), determine the amount of DOPA present originally and the amount remaining after oxidation by the tissue.

9. Subtract the amount of DOPA remaining after oxidation from that originally present in order to determine the amount of DOPA oxidized by the tissue.

10. Multiply the amount of oxidized DOPA by 60 in order to calculate the activity of DOPA oxidase, expressed as  $\mu$ g. of DOPA oxidized per Gm. of tissue per hr.

#### Calibration

1. Dilute the DOPA standard 1:10 in order to prepare a working standard (100  $\mu$ g. per ml.).

2. Pipet exactly 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 ml. of the working standard into a series of calibrated Klett tubes. Add barbiturate buffer to a final volume of 5 ml.

3. Incubate in a water-bath at 65 C. for maximal development of color as a result of auto-oxidation. The time required varies from 30 min., with concentrations of DOPA up to 100  $\mu$ g. per ml., to 2 hr. for 200  $\mu$ g.

4. Read absorbency (A) in a Klett-Summerson photoelectric colorimeter at 420  $m\mu$ .

5. Construct a standard curve by means of plotting the density of color (absorbency) in Klett units and the concentration of DOPA in  $\mu$ g. per ml. (Fig. 1).

#### RESULTS AND DISCUSSION

Pigment formed from DOPA yielded maximal absorption at 420  $m\mu$ ., and was stable for 1 hr. at 65 C. Addition of various amounts of ethyl alcohol or acetone, ranging to a final concentration of 20 per cent by volume, did not affect the amount of color subsequently developed. Decreasing the concentration of buffer to 0.01 M was also without measurable effect. With regard to these observations, it is of interest to note the finding of Riley and his associates,<sup>7</sup> namely, that the optimal concentration of phosphate buffer for the auto-oxidation of DOPA at 38 C. was between 0.1 M and 0.01 M.

On the other hand, formation of pigmented products in nonenzymatic oxidation of DOPA at 65 C. is affected by several factors, and the test conditions used in this study of the assay of DOPA were somewhat arbitrarily selected. The rate

of production of color increased with higher temperatures until the decreased concentration of dissolved oxygen became limiting. The concentration of oxygen seemed to be limiting at temperatures that were appreciably higher than 65 C., or with concentrations of DOPA in excess of 100  $\mu\text{g.}$  per 5 ml.; under such conditions, a dark ring was observed at the surface after quiescent incubation. Methods for increasing aeration were not generally used, however, inasmuch as the levels of DOPA in the studies described in this paper were not greater than 100  $\mu\text{g.}$  per 5 ml. With initial concentrations up to 100  $\mu\text{g.}$  of DOPA in 5 ml of buffer, the rate of production of color at 65 C. seemed to be constant until maximal color was attained. With greater concentrations of DOPA, the rate of formation of color was constant until the intensity was equivalent to that observed with 100  $\mu\text{g.}$  per 5 ml., after which the rate decreased appreciably, presumably because the concentration of dissolved oxygen became limiting. As stated above, maximal color was produced in 30 min. with up to 100  $\mu\text{g.}$  of DOPA per 5 ml., whereas a 2-hr. period of incubation was required with 200  $\mu\text{g.}$

The pH was an additional factor of importance in the rate of auto-oxidation of DOPA. With 50  $\mu\text{g.}$  of DOPA in 5 ml. of barbiturate buffer, no significant color was produced during 5 hr. at pH 4; however, maximal development of color occurred after 3 hr. at pH 6, or 20 min. at pH 10.

Homogenates of melanotic mouse melanoma, melanotic human melanoma, amelanotic human melanoma, or normal human muscle, liver, kidney, or spleen were incubated at 65 C., and there was no appreciable development of color, *i.e.*, these tissues contained no detectable DOPA. On the other hand, when known amounts of DOPA were added to any of the homogenates of tissue, the expected amount of color developed when the mixtures were incubated at 65 C., *i.e.*, added DOPA was fully recoverable.

In assays of DOPA in homogenates of tissue, clarification with acetone and centrifugation resulted in removal of most of the particulate matter, including the greater part of the color that developed from the enzymatic oxidation of DOPA and believed to be associated with particulate matter.<sup>6</sup> Even with highly pigmented melanomas, absorbency after this treatment generally was approximately 25, and never more than 50 Klett units. In agreement with the findings of Hogeboom and Adams,<sup>2</sup> and of Lerner and his associates,<sup>4</sup> nonenzymic oxidation of DOPA at 37 C., in the presence of tissues exposed to boiling temperature for 5 min., was comparable to that observed in the absence of tissue, and this effect was so small (<5 Klett units) that it could be disregarded.

The activity of DOPA oxidase in Cloudman mouse melanoma was maximal in homogenates with a 1:30 concentration of tissue. This amount of dilution facilitates the study of human tissues, inasmuch as specimens for biopsy may provide less than a gram of tissue. With 1:30 homogenates, the activity of DOPA oxidase was a function of the concentration of DOPA up to approximately 0.0006 M (120  $\mu\text{g.}$  per ml.); further increase in the concentration of DOPA did not result in increased activity. These findings are in virtual agreement with those of Riley and his associates,<sup>7</sup> who used extracts of Harding-Passey mouse melanoma. Although these workers observed enzymic oxidation that was proportional to concentrations of DOPA from 0.002 to 0.003 M, it would seem, from the content

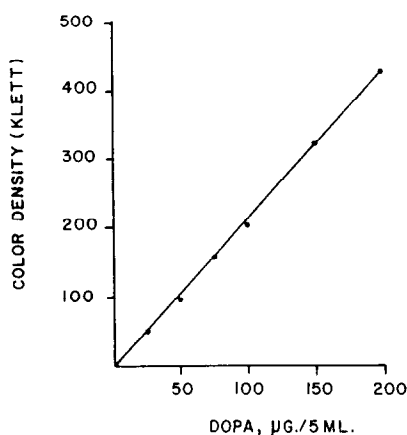


FIG. 1. Calibration curve illustrating the relation of the density of color to the concentration of DOPA (at 420  $m\mu$ ).

of nitrogen in their extracts, that the preparation of enzyme was possibly 4 times greater than that used in these studies. When *dl*-DOPA was used, the substrate ceased to be limiting at approximately twice the concentration required with *l*-DOPA, in confirmation of the reported strict specificity of DOPA oxidase for *l*-DOPA.<sup>9</sup>

A range of pH from 6.0 to 7.5 was satisfactory for the activity of DOPA oxidase. In confirmation of the work of Hogeboom and Adams,<sup>2</sup> and that of Riley and his associates,<sup>7</sup> enzymic oxidation was appreciably diminished at pH values less than 6, and auto-oxidation of DOPA at 37 C. was excessive at pH values greater than 8. With a 1:30 dilution of highly active tissue, oxygen became limiting after 30 min. of incubation at 37 C., unless the sample was aerated with a fine stream of air. Such aeration did not increase nonenzymic oxidation in heat-inactivated homogenates. The activity of DOPA oxidase did not seem to be affected after tissues were stored at  $-18$  C. for 6 months.

Typical values for the activity of DOPA oxidase in  $\mu\text{g. of DOPA removed per Gm. of tissue per hr.}$  were 1600 for Cloudman mouse melanoma, and 900 for human melanotic melanoma. Squamous cell carcinoma and normal human liver, kidney, spleen, muscle, or whole blood, did not manifest activity that was measurable.

It is thought that this method would be suitable for a study of the activity of DOPA oxidase in human melanomas. It should be emphasized, however, that the activity of DOPA oxidase in different portions of the same tumor may vary greatly, presumably as a result of the concentrations of tumor cells in the samples studied.

#### SUMMARY

This paper deals with a method for the colorimetric determination of the activity of DOPA oxidase in highly pigmented tissues. In order to facilitate the assay of added DOPA, and thereby increase the sensitivity of the determination

of the enzyme, most of the particulate matter and color in the preparations of tissues to be tested were removed by means of treatment with acetone and then by centrifugation.

#### SUMMARIO IN INTERLINGUA

Es describe un methodo pro le determination colorimetric del activitate de oxydase de DOPA in histos de alte grados de pigmentation. Pro facilitar le essayage de DOPA addite e augmentar assi le sensibilitate del determination del enzima, le plus grande parte del materia particulate e del color in le specimens de histo esseva eliminate per medio de un tractamento con acetona sequite per centrifugation.

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#### REFERENCES

1. GREENSTEIN, J. P., AND ALGIRE, G. H.: Comparative oxidase activity of melanotic and amelanotic melanomas. *J. Nat. Cancer Inst.*, **5**: 35-38, 1944.
2. HOGEBOOM, G. H., AND ADAMS, M. H.: Mammalian tyrosinase and DOPA oxidase. *J. Biol. Chem.*, **145**: 273-279, 1942.
3. HOROWITZ, N. H., AND SHEN, S. C.: Neurospora tyrosinase. *J. Biol. Chem.*, **197**: 513-520, 1952.
4. LERNER, A. B., FITZPATRICK, T. B., CALKINS, E., AND SUMMERSON, W. B.: Mammalian tyrosinase: Preparation and properties. *J. Biol. Chem.*, **178**: 185-195, 1949.
5. MARKERT, C. L.: The effects of genetic changes on tyrosinase activity in *Glomerella*. *Genetics*, **35**: 60-75, 1950.
6. MASON, H. S.: The structure of melanins. *In Pigment Cell Growth*. New York: Academic Press, Inc., 1953, pp. 277-303.
7. RILEY, V., HOBBY, G., AND BURK, D.: Oxidizing enzymes of mouse melanomas: Their inhibition, enhancement, and chromatographic separation. *In Pigment Cell Growth*. New York: Academic Press, Inc., 1953, pp. 277-303.
8. SPENCER, R. P., AND FIELD, J. B.: Quantitative colorimetric assay of tyrosinase substrates and inhibitors. *Proc. Soc. Exper. Biol. & Med.*, **88**: 576-578, 1955.
9. SUMNER, J. B., AND SOMERS, G. F.: *Chemistry and Methods of Enzymes*. New York: Academic Press, Inc., 1947, p. 309.