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## Dopa Oxidase Activity in Differential Diagnosis of Amelanotic Melanoma Tissue\*

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The histopathological diagnosis of melanomas with no demonstrable pigment is difficult. Such tumors are to be differentiated from anaplastic carcinomas, especially of the squamous and adenoid types, and from sarcomas showing marked anaplasia or spindle cells. In a previous report from this laboratory (1), a colorimetric method for determination of L-3,4-dihydroxyphenylalanine (dopa) oxidase activity in pigmented tissues was described, and it was suggested that a study of the enzymic oxidation of dopa might be of value in human melanomas. It is the purpose of this paper to present (a) an improved assay procedure and (b) studies of dopa oxidase activity in melanotic and amelanotic types of human melanoma tissue, other malignant tumors, and non-neoplastic tissues.

### MATERIALS AND METHODS

#### *Procedure for assay of dopa oxidase activity.*—

1. Tissues were obtained at the time of surgery or autopsy and placed immediately in a dry-ice cabinet until assayed.

2. Approximately 0.2 gm. of tissue was added to the tared tube of a Potter homogenizer and the exact weight determined.

3. Nineteen parts of distilled water and a small amount of silica sand No. 140 were added. With the homogenizer immersed in an ice bath, the tissue was thoroughly fragmented.

4. Ten parts of a stock 0.1 per cent solution of L-dopa (stored at  $-15^{\circ}\text{C}$ . in the dark and discarded if color developed) were added to yield

a 1:30 dilution of tissue containing 100  $\mu\text{g}/\text{ml}$  of dopa. One-ml. aliquots were dispensed into each of four to five test tubes ( $16 \times 150$  mm.). To two of the tubes serving as controls, 1 ml. of 10 per cent trichloroacetic acid was added immediately to inactivate the enzyme.

5. The two or three tubes containing active tissue were incubated at  $37^{\circ}\text{C}$ ., with vigorous agitation after 20 and 40 minutes. After exactly 1 hour of incubation, 1 ml. of 10 per cent trichloroacetic acid was added per tube to stop enzyme action and precipitate particulate matter and color thought to be associated with the particulates (5, 7).

6. Both test and control tubes were centrifuged for 10 minutes in an International Centrifuge No. 2 at 2000 r.p.m., and the clear supernatants were decanted into colorimeter tubes.

7. To each of the tubes, 4 ml. of 0.5 M sodium diethylbarbiturate (Merck) was added and the color density determined immediately in a Klett-Summerson photoelectric colorimeter at  $420\text{ m}\mu$ . The pH at this stage must be 9-10.

8. All tubes were incubated in a boiling water bath and read in the colorimeter at 5-minute intervals until two successive readings were essentially equal (10-15 minutes).

9. For each tube, the final colorimeter reading was subtracted from the initial reading to obtain the color density developed from dopa. The average color density produced by the tubes incubated for enzymic oxidation was then subtracted from the average color density produced by the controls, and the amount of dopa oxidized by the tissue was determined from a standard curve obtained by plotting color density vs. dopa concentration in  $\mu\text{g}$ . (1). This value was multiplied by the tissue dilution factor of 30 and divided by 1000 in order to express the data as mg. of dopa oxidized per gm. of tissue in 1 hour. There was a linear relation between color density and dopa concentration over the range employed (up to 100  $\mu\text{g}$ . of dopa/ml).

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*Tissues.*—In total, 55 tissues were assayed, distributed as follows: melanotic melanoma tissues, fourteen; amelanotic melanoma tissues, eight; malignant tumors other than melanoma, twelve; and non-neoplastic tissues, 21. Histopathological classification of the melanomas as to presence or absence of pigment was made only after painstaking examination of paraffin sections stained with hematoxylin and eosin, with multiple sections studied when necessary.

Tissues were obtained at the time of surgery or autopsy and stored in a dry-ice cabinet until assayed. It was estimated that specimens secured at autopsy were obtained 4–6 hours after death. The malignant tumors were trimmed of grossly normal and/or necrotic tissue, and histopathological sections were prepared from tissue adjacent to samples taken for assay of dopa oxidase activity. In the melanotic tissues, the concentration of pigment varied widely. Some tumors were grossly black, while in others pigment was detected only after careful examination of all available sections. In one patient, a grossly black metastatic tumor mass was included among the melanotic tissues, while a separate nonpigmented metastasis from the same patient was included in the amelanotic melanoma category.

Malignant tumors other than melanoma included two adenocarcinomas of breast; one each of adenocarcinoma of cervix, adenocarcinoma of stomach, adenocarcinoma of prostate, alveolar cell carcinoma of lung, oat-cell carcinoma of lung, reticulum-cell sarcoma, and squamous-cell carcinoma of skin; and two unclassified malignant tumors. Some of the tissues assayed were from primary tumors, while others represented metastatic lesions.

Non-neoplastic tissues assayed were obtained from autopsies of three adults, with causes of death designated, respectively, as massive infarction, hemochromatosis, and Hand-Schuller-Christian disease, and from one infant of 2 months who died of pneumonia. Tissues studied included liver from all four individuals; adrenal, brain, and spleen from three; renal cortex from two; and a bone lesion (Hand-Schuller-Christian disease), diaphragm, heart muscle, lung, thymus, and thyroid, each from one of the four individuals. Eight of the tissues were obtained from one individual, six from the second, four from the third, and three from the fourth.

*Replication.*—Each datum in Chart 1 represents at least one assay of one tissue homogenate, the value shown being the average of duplicate or triplicate tubes. In the melanotic melanoma tissues, two of the values are means of assays of

two separate homogenates, one the mean of four, and one the mean of five. For the amelanotic melanoma tissues, two values represent the average of assays of two homogenates, and two the averages of four. In the malignant tumors other than melanoma, one value is the mean of assays of three homogenates.

## RESULTS AND DISCUSSION

The data are presented in Chart 1 in terms of mg. of dopa oxidized/gm of tissue in one hour. With the fourteen melanotic melanoma tissues, assay values ranged from 0.22 to 2.4; the eight amelanotic melanoma tissues exhibited assay levels from 0.12 to 0.96. The malignant tumors other than melanoma and the non-neoplastic tissues showed values from 0.0 to 0.08. It is felt that the tissues other than melanoma actually contained

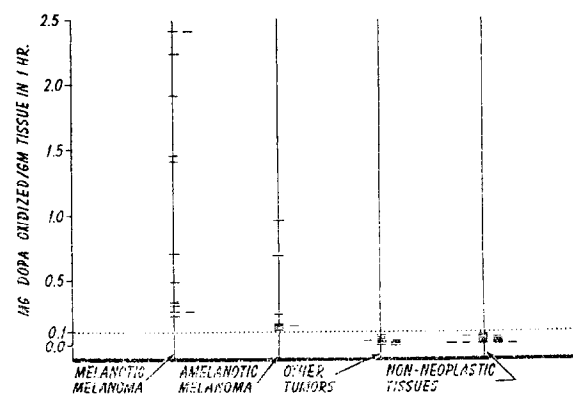


CHART 1.—Dopa oxidase activity of human tissues

no dopa oxidase and that the values obtained fall within the range of variation inherent in the assay procedure. At any rate, the data obtained allowed differentiation, in the tissues studied, between the melanomas and the other tissues, neoplastic or not. The arbitrary dotted line in Chart 1 is included to facilitate visualizing this differentiation. Even if further investigation were to reveal amelanotic melanoma tissues without demonstrable dopa oxidase activity, the *presence* of such activity would be of value in the differential diagnosis of melanomas without demonstrable pigment. For example, the two unclassified malignant tumors included in the group of tumors other than melanoma might well have been designated amelanotic melanomas had they exhibited appreciable dopa oxidase activity.

Apart from possible practical application in differential diagnosis, the significance of the oxidation of dopa by amelanotic melanoma tissue is by no means certain. It may be postulated that

the complete enzyme system leading to the production of melanin from tyrosine (5) is present in amelanotic tumor tissue but in amounts so small that the pigment produced does not accumulate in sufficient concentration to be detected by the usual microscopic examination. However, this explanation seems inadequate in view of the finding that two of the amelanotic melanoma tissues exhibited higher dopa oxidase activity than seven of the melanotic tumors. An inhibitor of melanin production, effective *in vivo* but inactivated in the assay by dilution or other means, appears plausible in view of the finding that dopa oxidase activity calculated on the basis of a tissue dilution of 1:30 was appreciably greater than that obtained from a 1:15 dilution. There is evidence that sulfhydryl groups function as such an inhibitor in normal skin, being inactivated by ultraviolet irradiation through oxidation (5). A third explanation is conceivable. Mammalian tyrosinase, the enzyme oxidizing tyrosine to dopa, has generally been considered as distinct from dopa oxidase (1, 2, 5), although this view has been challenged (5). If the two enzymic stages in melanin formation are mediated by separate enzymes, tyrosinase might be postulated to be absent from amelanotic melanoma tissue. Indeed, with microspectrophotometric-autoradiographic methods, Speece *et al.* (8) failed to detect tyrosinase activity in amelanotic portions of melanomas. Since dopa is not a normal tissue constituent (1, 5), melanin would not be produced *in vivo*. In this connection, it is of interest that cytochemical production of melanin in normal skin by the Bloch technic is positive only when tissue sections are incubated with dopa, and it is uniformly negative when tyrosine is used as the substrate (5).

The method for determination of dopa oxidase activity differs in three respects from that previously published (1). Decreasing the dopa concentration from 200 to 100  $\mu\text{g}/\text{ml}$  decreases the amount of aeration necessary for the enzymic oxidation, so that vigorous agitation at 20-minute intervals can be used in place of constant aeration with a fine stream of air. Trichloroacetic acid is used in place of acetone to stop enzyme action and precipitate particulate matter and melanin believed to be associated with it (5, 7). With this reagent, water-clear supernatants are nearly always obtained, with only an occasional assay showing a tinge of color prior to auto-oxidation. Finally, the incubation for auto-oxidation is carried out at 100° C. rather than at 75° C. This procedure results in more rapid oxidation, with

the time required for completion of the process reduced from 30 to 10–15 minutes.

With two exceptions, assays of replicate homogenates agreed within  $\pm 10$  per cent. However, assays of four homogenates of one amelanotic melanoma tissue exhibited a range of 0.56–0.85 mg. of dopa oxidized/gm of tissue, and assays of two homogenates of a second amelanotic tumor yielded values of 0.08 and 0.16. Such unusual spreads may reflect inherent variability in enzyme content of different portions of the tumors, particularly since replicate assays on a single homogenate invariably agreed rather closely.

Due allowance must be made for the instability of both dopa and dopa oxidase. The maximum color density developed by auto-oxidation of 100  $\mu\text{g}$ . of fresh dopa varied from 250 to 300 Klett units, depending on the source, while dry dopa kept at room temperature for 1 year showed less than 100 Klett units. However, stock 0.1 per cent solution may be preserved in the frozen state at  $-15^{\circ}\text{C}$ . for many months. Tissues left at room temperature for 24 hours showed complete loss of dopa oxidase activity; therefore, unless tissues are assayed immediately, they should be frozen without delay. In tightly closed containers, tissues have been stored for 6 months at  $-15^{\circ}\text{C}$ . and for 18 months in a dry-ice chest without demonstrable reduction in dopa oxidase activity.

#### SUMMARY

By means of an improved assay procedure, dopa oxidase activity was present in all of fourteen melanotic melanoma tissues and eight amelanotic melanoma tissues. It was felt that the assay values obtained with twelve malignant tumors other than melanoma and 21 non-neoplastic tissues were within the range of variation inherent in the assay method. It would appear that dopa oxidase activity may be of value in the differential diagnosis of melanomas without demonstrable pigment. Possible explanations of the presence of dopa oxidase activity in amelanotic melanoma tissue are discussed.

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