An Enzymatic and Electron Microscopic Characterization of a Variant of the Cloudman S-91 Melanoma*

HERBERT M. HIRSCHT AND ALVIN S. ZELICKSON

(Department of Pathology and Division of Dermatology, University of Minnesota School of Medicine, Minneapolis, Minnesota)

SUMMARY

A variant of the Cloudman S-91 melanoma is described which is characterized by a low level of tyrosinase and dopa oxidase (phenolase complex) activity. On the ultrastructural level this is expressed by a preponderance of melanosomes (immature melanin granules) rather than mature melanin particles. Extracts of the tumor exhibit only dopa oxidase activity. With tumor slices, tyrosinase activity can be demonstrated as well; catalytic amounts of dopa have a stimulating effect on this reaction. Dopa oxidase activity is competitively inhibited by tyrosine.

Inter-tumor variation with respect to melanogenesis is correlated with the presence, in some tumors, of a potent inhibitor of tyrosinase and dopa oxidase. The inhibitor can be demonstrated directly in enzymatic tests by mixing active and inhibitory extracts. Selection experiments showed that control of this inhibitor resides within the tumor rather than the host.

With respect to both phenolase complex activity and ultrastructural characteristics, it can be concluded that this tumor is a genetic variant of the original S-91 melanoma and occupies a position intermediate between the melanotic and the amelanotic variety. The development and the relationship to the endoplasmic reticulum of type A particles within this tumor are described.

The Cloudman S-91 melanoma is a pigmented tumor which produces rather large amounts of melanin via the tyrosinase-dopa oxidase (phenolase) complex of enzymes (15). Amelanotic variants have been obtained in selection experiments (1, 16). Such variants have been reported to produce neither tyrosinase nor dopa oxidase (15). Another variant of the Cloudman S-91 melanoma has recently been obtained by Foley et al. (9) and Foley1 by carrying S-91 melanoma cells in tissue culture and then reintroducing the cells to the DBA mouse from which the tumor originally was obtained. Foley1 has described the tissue culture-derived melanoma as being an amelanotic variant. Sloboda and Kopac (42) found the S-91 cells in tissue culture to be devoid of melanin and dopa oxidase.

We have recently had occasion to examine this tissue culture-derived melanoma in detail and found it to possess a number of interesting properties. The present report will describe some of these characteristics which suggest that the present tumor represents a genetic variant of the original S-91 tumor, with biochemical and ultrastructural characteristics which place it between the melanotic and the amelanotic variety.

MATERIALS AND METHODS

Tumor and animals.—The S-91 melanoma variant (Foley) used in these experiments was obtained from Microbiological Associates, Inc., Bethesda, Md., as a transplant carried in MAI-DBA/2 female mice. The tumor line carried the designation S-91 III/2 and had been re-established in mice following prolonged passage of the S91 cells in tissue culture (Foley et al. [9]; Foley1). The tumor was maintained in our laboratory by serial subcutaneous trocar transplantation into female DBA/2 (Bi) strain mice at approximately 3- to 3½-week intervals.

Enzymatic assays.—The phenolase complex activity of this tumor was so low that a photometric method for measuring its activity was used. Tyrosinase and dopa oxidase activities2 were determined by measuring the amount of melanin formed at approximately 430 mμ, with

1 The terms tyrosinase and dopa oxidase will be employed, depending on whether tyrosine or dopa are used as the respective substrate. This is done in accordance with the convention of naming an enzyme in terms of the reaction catalyzed, and without implication as to the unitarian or dualistic nature of this enzyme. When both activities are referred to as part of a single enzyme or enzyme complex, the term phenolase complex will be used.


† Research Career Development Awardee (5-K3-GM-2634) of the National Institute of General Medical Sciences, Public Health Service.

1 G. E. Foley, personal communication.

Received for publication December 12, 1963.
tyrosine or dopa used as a substrate. This method is considerably more sensitive than the manometric method; similar methods have been used extensively in investigations of fungal tyrosinases (17, 21) and in assays of mammalian tyrosinase (3, 41). The method, although very sensitive and reproducible, has some disadvantages (10). It was, however, the one most feasible here owing to the very low enzyme activities found to be present. Use of this assay was justifiable, because melanin solutions so formed (if measured prior to precipitation) follow (18) the laws of Beer and Lambert over a wide range of concentration and because melanin formation was found to be proportional to enzyme extract. It should be noted also that melanin formed by dopa autoxidation (controls) absorbs light in a manner similar to melanin formed enzymatically (18).

A Klett-Summerson photoelectric colorimeter, Klett photometer tubes, and No. 42 blue filter were used; optical density (O.D.) is expressed in Klett units (K.U.). The usual assay system consisted of tumor extract, M/10 phosphate buffer, pH 6.8, and L-tyrosine (Nutritional Biochemicals Corp.) or L-dopa (3,4-dihydroxyphenyl-L-alanine) (Nutritional Biochemicals Corp.) as substrate, in a total volume of 6.0 ml. Incubation temperature was 37° C. Tyrosine was dissolved in M/60 NaOH, dopa in M/10 phosphate buffer, pH 6.8, both at a concentration of 2 mg/ml of solvent.

A typical assay mixture consisted of between 0.2 and 0.4 ml of tumor extract, 1.0 ml of tyrosine or dopa (2 mg/), and phosphate buffer to a final volume of 6.0 ml. Dopa is an autoxidizable substrate, and appropriate dopa autoxidation controls were always run. Both tests and controls were usually determined in triplicate. Tubes were frequently shaken by hand to maintain oxygen equilibrium. Modifications of the procedure will be indicated in the text.

Preparation of tumor extracts.—Tumors were dissected out, care being taken to take only non-neoplastic tumor tissue. The tumors were weighed, finely minced with scalpels in a Petri dish containing a little glucose-potassium chloride-sodium chloride solution and ground thoroughly in a mortar and pestle with 5 times their weight of ice-cold w/10 phosphate buffer, pH 6.8. The ground suspension was filtered through several layers of gauze and the filtrate centrifuged for 10 minutes at 800 r.p.m. The supernatant was used as the tumor extract, and either used immediately or stored at −20° C. for further use. All steps in the procedure were done with aseptic precautions.

Protein was determined by a modification (36) of the method of Lowry et al. (30), with crystalline bovine plasma albumin (Armour) used as a standard, or by a micro-Kjeldahl procedure.

Electron microscopy.—The tumors used for electron microscopy were harvested 27-39 days following implantation. The tumor tissue was cut into 1-mm. cubes and immediately fixed in 1 per cent buffered osmium tetroxide. The cubes were embedded in Epon 812 and sectioned with an LKB ultrotome. Thin sections were stained for 30 minutes with uranyl acetate and studied with an RCA EMU 3F electron microscope.

RESULTS

Morphology.—The present melanoma varies in color, from animal to animal, from light to black. Distinct differences in color may occur within the same tumor. Foley ascribed the amelanotic character of the tumor (inability to produce melanin in enzymatic tests, as contrasted with gross observation of tumor pigmentation) to the loss of melanocytes resulting from repeated passage of the tumor in tissue culture. However, microscopic examination of this tumor showed the presence of very numerous melanocytes, with pigment present in both the neoplastic cells and macrophages. Its amount varied greatly in the tumors studied and in different areas of the same tumor. The pigment stained with ammoniacal silver solution.

Electron microscopy.—The nuclei of the tumor cells are round, large, and granular. The mitochondria are filamentous, abundant, and scattered throughout the cytoplasm (Fig. 1). The endoplasmic reticulum is rough-walled, well developed, and in a dispersed state (Fig. 1). Ribosomes are also abundant throughout the cytoplasm. The cell membranes are relatively straight and closely approximate one another. Desmosomes are few in number and are poorly developed (Fig. 1). The Golgi complex is hypertrophied and located about the nucleus. Melanosomes (40) were found to be present and located in the region of the Golgi apparatus. They vary in size from approximately 0.2-1.0 μ in diameter and are enclosed by a smooth-walled membrane (Fig. 1). Often a striated membrane is located in the center of the organelle. Each striation on the membrane is separated from its neighbor by a 90 Å clear zone. Mature melanin granules are also present but are few in number (Fig. 1). They also range in size up to 1.0 μ in diameter.

![Chart 1](chart1.png)

**Chart 1**—Determination of tyrosinase and dopa oxidase in extracts from Cloudman S-91 melanoma variant. Age of tumors, 26 days. Protein content of tissue preparation used, 6.7 mg/ml. Substance and other additions as indicated under “Materials and Methods.”

- Curve A: dopa and 0.4 ml of tissue preparation.
- Curve B: dopa and 0.2 ml of tissue preparation.
- Curve C: dopa autoxidation control.
- Curve D: tyrosine and either 0.2 or 0.4 ml of tissue preparation.
Tyrosinase and dopa oxidase determinations with tumor extracts.—Biochemically, the Cloudman S-91 melanoma is characterized by the presence of both tyrosinase and dopa oxidase, whereas the amelanotic (S-91 A) derivative has neither of these activities (15, 16). The present variant, as judged by extracts prepared by the relatively very mild procedures used here, was characterized by a complete absence of tyrosinase and a very low level of dopa oxidase activity (Chart 1).

Tyrosinase in melanoma is present in an active state (28) and, in contrast to tyrosinase ("cresolase") activity in mushroom and potato preparations, has been found to be relatively stable (32). Activation of initially inactive mammalian tyrosinase preparations can be achieved through the addition of small amounts of dopa (29). It has also been suggested that the tyrosine-tyrosinase reaction can be accelerated with certain pituitary gland preparations ([11]; see however [26]) and that adrenocorticotropic hormone activates an enzyme system or releases an inhibitory mechanism of pigment formation (22).

An attempt was therefore made to activate the tyrosinase activity in the extracts by various means, including priming the enzyme in the presence of tyrosine through the addition of catalytic amounts of dopa (Chart 2), through addition of adrenocorticotrophin in vitro, as well as by prolonged incubation (up to 3 days). None of these methods was successful. In fact, tyrosine inhibited dopa oxidase when small amounts of dopa were present (Chart 2).

Variability in dopa oxidase activity in different tumors and its relation to an inhibitor of melanogenesis.—Extracts were prepared with, as a starting material, larger pools of tumors. Surprisingly, preparations made from such pools showed

The differences in the readings between tissue + dopa (small amount) and tissue + dopa (small amount) + tyrosine were statistically significant (e.g., .02 > P > .01 for the last reading).
neither tyrosinase nor dopa oxidase activities, and frequently had present an inhibitor of dopa-oxidase and dopa autoxidation (Chart 3). With tumor extract $\#3$, inhibition was approximately 30-40 per cent throughout when compared with the dopa autoxidation control. When smaller amounts of dopa were used (.05 mg. instead of 2 mg.), inhibition of dopa oxidation was essentially complete.

To determine the reasons for the variability in extracts made from pools, a number of individual tumors were assayed. Such assays showed that each tumor had its own individual dopa oxidase activity, as seen in Chart 4. Individual tumors exhibited three different phenotypes: (a) definite dopa oxidase activity, (b) no enzyme activity, or (c) presence of an inhibitor of dopa oxidase and dopa autoxidation. These results clearly show that absence of enzymatic activity in extracts made from pools of tumors was due to the presence, in some tumors, of a potent inhibitor of dopa oxidase and dopa autoxidation. This was further corroborated when it was shown that prolonged storage of the extracts (even at $-20^\circ$ C.) inactivated the inhibitor and permitted initially inhibited preparations to regain dopa oxidase activity (Chart 5). An inhibitor of dopa autoxidation in a number of nonmelanotic tissues has been described previously (19); that inhibitor differs from the present one, however, because it was not inactivated on storage. This phenomenon will be discussed later.

Presence of the inhibitor could be demonstrated directly in experiments in which enzymatically active and inhibitory extracts were mixed. Such an experiment is reported in Table 1. It is clear from this table that the inhibitory extract contains a potent inhibitor of dopa autoxidation and that it inhibits the dopa oxidase activity of the active extract.

No correlation between tumor weight and melanogenesis was observed in assays on individual tumors (see Chart 4), but a definite correlation between gross tumor pigmentation and actual dopa oxidase activity in the extracts was observed—i.e., the more pigmented the tumor, the greater was the dopa oxidase activity. It is obvious from such correlations that the inhibitor is physiologically active in vivo and that its presence or absence determines to a large extent the actual degree of melanization of the tumors.

The present data show that implants derived from a single tumor progenitor can give rise to tumors that vary tremendously in their enzymatic makeup (with respect to melanogenesis) in the recipient inbred mice. This variability is due to the presence of an inhibitor in some, but not all, of the tumors. Two factors could be responsible for this variability: (a) host factors or (b) genetic variability in the cells comprising the tumor inocula. To differ-
CHART 5.—Reactivation of initially inhibited dopa oxidase preparations following storage at low temperature. Protein content of tumor tissue preparations: extract #2, 8.6 mg/ml; extract #3, 7.5 mg/ml. Amount of extract used: 0.3 ml/tube. Substrate and other additions as indicated under “Materials and Methods.” Period of storage of the two extracts at −20°C: 5 and 4 months, respectively.

TABLE 1

DIRECT DEMONSTRATION OF THE PRESENCE OF THE INHIBITOR OF DOPA AUTOXIDATION AND DOPA OXIDASE ACTIVITY IN EXTRACTS PREPARED FROM NONPIGMENTED TUMORS

“Active extract” was prepared as usual from a pigmented tumor, 57 days old; the extract contained 8.4 mg protein/ml. “Inhibitory extract” was prepared from nine nonpigmented tumors, 15–21 days old; it contained 9.7 mg protein/ml. Assay conditions were as outlined under “Materials and Methods.” Readings given in the table are the means from closely checking duplicate or triplicate determinations, following incubation for 90 minutes at 37°C.

<table>
<thead>
<tr>
<th>Addition</th>
<th>O.D. (Klett units)</th>
<th>Per cent inhibition of “active extract” control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopa only (autodxidation control)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Dopa + active extract (0.3 ml.)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Dopa + inhibitory extract (0.1 ml.)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot; + &quot; (0.3 ml.)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&quot; + &quot; (0.6 ml.)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dopa + active extract (0.3 ml.) + inhibitory extract (0.1 ml.)</td>
<td>16 24</td>
<td></td>
</tr>
<tr>
<td>&quot; + &quot; (0.3 ml.) + &quot; (0.3 ml.)</td>
<td>12 43</td>
<td></td>
</tr>
<tr>
<td>&quot; + &quot; (0.3 ml.) + &quot; (0.6 ml.)</td>
<td>5 76</td>
<td></td>
</tr>
</tbody>
</table>

To differentiate between these two possibilities a simple selection experiment was done in which small amounts of tissue from a pigmented and from an unpigmented tumor were selected and inoculated into DBA/2 mice. All tumors derived from the inocula of the pigmented tumor gave rise to pigmented tumors, whereas (with one exception) all the tumors derived from the nonpigmented tumor showed little pigmentation. Results of dopa oxidase assays on a number of these tumors are shown in Chart 6.

Although there is some overlap, it is clear that tumors deriving from the pigmented tumor progenitor have the highest dopa oxidase activity, whereas two tumors derived from the relatively nonpigmented tumor progenitor are the only ones that have an inhibitor of dopa oxidase and
dopa autoxidation present. This experiment shows that control of melanogenesis, at least under the experimental setup used here, resides to a large extent within the tumor and not within the host; it indicates the extensive genetic variability within the melanocytes comprising the tumor and shows the correlation existing between the characters "melanotic" or "amelanotic" on one hand and the presence or absence of the inhibitor of the phenolase complex on the other. Undoubtedly, there may exist situations where the environment retains a larger share of the control of tumor pigmentation (local oxygen tension, substrate level, etc.). More rigorous experiments on the interplay of environment and heredity in this system would necessitate the use of tumors derived from single cell inocula. Such experiments, in which a different melanoma was used, were performed by Gray and Pierce (14), with results entirely analogous to those obtained here. The present findings with the S-91 variant are also essentially in agreement with those of Hesselbach (16) and Algire et al. (1), who found the control of pigmentation to reside largely within the tumor, rather than the host. The present data extend the findings of Algire et al. and Hesselbach, relating them directly to the inhibitor level.

Tyrosinase and dopa oxidase activities in intact tumor slices.—As has been shown, extracts of the tumor were unable to convert tyrosine to dopa but did convert dopa to melanin. The presence of dopa oxidase activity, as contrasted with the absence of tyrosinase activity, raises a number of questions. First, how is the relatively small amount of melanin found in the tumor formed? Second, is dopa oxidase an enzyme separate from tyrosinase?

Possible answers to these questions are: (a) melanin is formed in the tumor via dopa oxidase only, acting on diphenols formed elsewhere in the tissues of the host; (b) melanin is formed by the conventional phenolase complex, but the tyrosinase activity is destroyed during the preparation of the extract.

To answer these questions, experiments on melanogenesis were undertaken with the use of intact tumor tissue rather than extracts. Small pieces of each tumor were put on filter paper pads in Petri dishes and bathed in a fluid containing either: phosphate buffer + tyrosine; buffer + tyrosine + a catalytic amount of dopa; buffer + dopa; or buffer alone. A number of appropriate controls without tissue were also included. The experiments were run under aseptic conditions; in addition to these precautions, antibiotics (penicillin and streptomycin) were added to the liquid medium.

The Petri dishes were incubated for several days at 37°C in a humidified incubator, and the amount of melanin formed in the bathing fluid was determined photometrically at the termination of the experiment. This procedure gave excellent results; on the other hand, observation of increases in pigmentation in the tumor slices proved to be worthless. Critical factors affecting the experiments were found to be: (a) depth of fluid surrounding the suspended tissue slice ("drowning" of the tissue had to be avoided) and (b) age of the tumors.

Results of four separate experiments involving seventeen individual tumors can thus be summarized: After extended periods of incubation, tyrosine is converted to melanin, which is released into the liquid. The reaction is relatively slow. It is clear, therefore, that the tumor can convert tyrosine to dopa but that this tyrosinase activity of the phenolase complex is bound to the intact cell and is destroyed in the relatively very mild process of preparing the enzyme extract. Representative results are given in Table 2.

Tyrosinase activity in many of the tumors could be stimulated extensively through the addition of catalytic amounts of dopa; two-thirds of all tumors tested showed this effect. In these, dopa, in addition to acting as a substrate, acts as an activator of tyrosinase. In general, stimulation of tyrosinase by catalytic amounts of dopa occurred only in the less pigmented tumors. These results may indicate that, in the more heavily pigmented tumors, tyrosinase activity is at a maximum and that the local O/R potential is of considerable importance in determining in vivo tyrosinase activity.

Tyrosinase activity (Table 2) can be demonstrated in older (35- to 49-day-old), but not in the more recently
### TABLE 2

**MELANOGENESIS BY SLICES OF THE S-91 MELANOMA VARIANT**

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Tumor no.</th>
<th>Age of the tumor used (days)</th>
<th>Gross tumor pigmentation</th>
<th>Period of incubation of slices at 37°C (hr.)</th>
<th>Substrate*</th>
<th>Melanin formation (Net O.D. readings) K.U.†</th>
<th>Wet weight of tissue slice (mg.)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>9B</td>
<td>1</td>
<td>46</td>
<td>Greyish black</td>
<td>96</td>
<td>Tyrosine</td>
<td>26</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>99</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td>9B</td>
<td>2</td>
<td>46</td>
<td>Grey</td>
<td>96</td>
<td>Tyrosine</td>
<td>7</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>57</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>9B</td>
<td>2a</td>
<td>46</td>
<td>Grey</td>
<td>96</td>
<td>Tyrosine</td>
<td>9</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>118</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>9B</td>
<td>4a</td>
<td>46</td>
<td>Black</td>
<td>96</td>
<td>Tyrosine</td>
<td>110</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>158</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>72</td>
<td>50</td>
</tr>
<tr>
<td>9B</td>
<td>5</td>
<td>18</td>
<td>Unpigmented to light grey</td>
<td>96</td>
<td>Tyrosine</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>59</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>9C</td>
<td>1</td>
<td>49</td>
<td>Black</td>
<td>72</td>
<td>Tyrosine</td>
<td>164</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>257</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>76</td>
<td>161</td>
</tr>
<tr>
<td>9C</td>
<td>2</td>
<td>49</td>
<td>Unpigmented to light grey</td>
<td>72</td>
<td>Tyrosine</td>
<td>28</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>43</td>
<td>74</td>
</tr>
<tr>
<td>9C</td>
<td>5</td>
<td>24</td>
<td>Unpigmented</td>
<td>72</td>
<td>Tyrosine</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine + dopa</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dopa</td>
<td>45</td>
<td>24</td>
</tr>
</tbody>
</table>

* The following substrates were used: tyrosine, 0.5 ml. containing 1 mg. of L-tyrosine; tyrosine + dopa, 0.5 ml. containing 1 mg. of L-tyrosine and 0.02 ml. containing 0.04 mg. of L-dopa; dopa, 0.02 ml. containing 0.04 mg. of L-dopa. Controls containing no substrate also were run.

† The volume in each Petri dish was brought to 2.0 ml. with 1/10 phosphate buffer, pH 6.8. In addition, penicillin and streptomycin were present at a concentration of 100 units or µg each, respectively, per ml. of liquid medium. Each tissue slice was suspended on a filter paper pad, 1 cm. square, in a small (60 X 15 mm.) Petri dish.

‡ “Net O.D. readings” implies that the reading of the “tissue control” (tissue slice bathed by 2.0 ml. of fluid but without substrate) has been deducted.

Optical density readings were done as outlined under “Materials and Methods,” with the exception that, at the termination of the experiment, the fluid from each Petri dish was carefully washed into a Klett tube, brought to exactly 5.0 ml. with distilled water, and then read.

The pH was measured on all supernatant fluids at the termination of the experiments and following the photometric readings. It remained constant (mean pH on all samples, 6.9; range, 6.8-7.0).

Wet weights of the tissue slices were determined at the end of the experiments. All slices were blotted on filter paper prior to weighing to remove extraneous moisture.

Inoculated (18- to 26-day-old) tumors. It is apparent further that tyrosinase activity is directly related to pigmentation of the tumors and, judging from the experiments presented in a previous section of this paper, it seems probable that the low activity is directly related to the presence of an inhibitor of melanogenesis in the tumor. The data indicate a decrease in inhibitor concentration with increasing age of the tumor. As found previously with dopa oxidase, no correlation between tumor size and tyrosinase activity was observed.

The very slow rate of conversion of tyrosine and dopa to melanin was further confirmed in an electron microscopic-autoradiographic study of melanogenesis, with triitated tyrosine and dopa used as substrates. These experiments were carried out in collaboration with A. S. Zelickson, H. M. Hirsch, and J. F. Hartmann, localization of melanin synthesis within the pigment cell: determination by a combination of electron microscopic autoradiography and topographic planimetry. (Submitted for publication.)

---

* Cited references are from the original text.
within this tumor is destroyed in the course of preparation of the enzyme, whereas dopa oxidase activity remains apparently not protected in the highly organized macroroma is easily inactivated is further evidence that it is site of the phenolase complex molecule, it was of interest that tyrosinase and dopa oxidase ("cresolase" and "catecholase") activities are associated with the same active site of the phenolase complex molecule, it was of interest to determine the behavior of dopa oxidase in the presence of both dopa and tyrosine, to ascertain whether tyrosine inhibits dopa oxidase and whether such inhibition is competitive or noncompetitive. If inhibition is competitive, it would indicate that substrate (dopa) and inhibitor (tyrosine) compete for the same site on the enzyme.

To determine the type of inhibition, the method of Dixon (6) was employed; it involves rate measurements at two substrate concentrations in the presence of various inhibitor concentrations. The system used here necessitates the use of a number of additional controls; a dopa autoxidation control is needed at every inhibitor level, in view of the possibility that tyrosine inhibits dopa autoxidation as well as enzymatic dopa oxidation. In order to circumvent complications introduced by the presence of the tissue inhibitor of melanogenesis, a tissue preparation stored for a number of months at -20° C. was used; in such preparations, the inhibitor has been inactivated (see above).

Results (Chart 7) show that tyrosine inhibits dopa oxidase. Inhibition is substrate-dependent—i.e., it is much greater at low (upper curve) than at high (lower curve) concentrations of dopa and is thus competitive. It is well known that, in the case of competitive inhibition, substrate as well as inhibitor competes for enzyme surface and that, therefore, changes in the concentration of either will influence the degree of inhibition observed. On the other hand, substrate concentration has no influence in cases of noncompetitive inhibition. A Dixon plot (6), in which the reciprocals of the initial velocity were plotted against the reciprocals of the initial velocity, did not yield additional information.

With the lower concentration of dopa, tyrosine, at higher concentrations, also inhibited the process of dopa autoxidation, but to a much smaller extent than enzymatic dopa oxidation. This was not surprising, in view of previous findings (18) that a number of amino acids give slight inhibition of dopa autoxidation—a phenomenon ascribed to the possibility that the amino acids chelate traces of copper or other ions needed for the catalysis of the autoxidation.

Correlation between biochemical findings and ultrastructure.—In the present tumor the main block in melanogenesis lies in its reduced capacity to convert tyrosine to dopa. It was of interest to determine how this phenomenon expresses itself on the ultrastructural level. Melanotic melanomas are characterized by the presence of many mature melanin granules, amelanotic melanomas by the presence of pre-melanosomes or melanosomes—i.e., immature melanin particles (43, 45). In the S-91 melanoma, the number of nonpigmented stages in the formation of the melanin particles is relatively low (33), suggesting a rapid rate of melanization. On the contrary, the present S-91 melanoma variant has a preponderance of melanosomes and only relatively few mature melanin granules. It is, therefore, intermediate between the melanotic and the amelanotic type of melanoma (Figs. 1, 2).

Presence of type A particles in the tumor.—Also seen in Figure 1 are a number of electron-dense, osmiophilic particles. Following their outgrowth from the rough-walled endoplasmic reticulum (Fig. 3), these vesicles eventually

---

**Chart 7.—Inhibition of dopa oxidase by tyrosine.** Protein content of tissue extract used: 8.6 mg/ml. Amount of extract used per tube: 0.3 ml.

All readings are means from duplicate determinations. The values recorded on the chart are "net-readings"—i.e., each sample at every concentration of substrate and inhibitor had its own dopa autoxidation control, and these values were deducted from the readings obtained in the presence of enzyme. Dopa was used at two concentrations, 0.1 ml. or 1.0 ml. per tube (final concentration 1.7 X 10^{-4} M, or 1.7 X 10^{-6} M), and labeled "low substrate concentration" and "high substrate concentration," respectively, on the chart. Inhibitor (tyrosine) was used at three or four concentrations:

- **F**, no tyrosine;
- **G**, 0.1 ml. tyrosine per tube, final concentration 1.84 X 10^{-4} M;
- **H**, 0.5 ml. tyrosine per tube, final concentration 9.2 X 10^{-4} M;
- **I**, 1.0 ml. tyrosine per tube, final concentration 1.84 X 10^{-3} M;
- **K**, 1.5 ml. tyrosine per tube, final concentration 2.76 X 10^{-3} M.

The final pH was checked on all samples at termination of the experiment and found to be approximately 6.85 in all tubes.

Experiments showed very slow incorporation of tyrosine and dopa into melanin in the melanosomes, the supposed site of activity of tyrosinase and melanogenesis within the melanocytes. On the contrary, most of the label was found to be present in the endoplasmic reticulum, suggesting the presence of phenolase complex activity there. The fact, shown above, that tyrosinase in the present melanoma is easily inactivated is further evidence that it is apparently not protected in the highly organized macrostructure of the melanin granule (32).

Competitive inhibition of dopa oxidase by tyrosine and the active site of the mammalian phenolase complex molecule.—As shown above, the small amount of tyrosinase activity within this tumor is destroyed in the course of preparation of the enzyme, whereas dopa oxidase activity remains active. It was also suggested (see Chart 2) that tyrosine inhibited dopa oxidase. In view of the hypothesis (32) that tyrosinase and dopa oxidase ("cresolase" and "catecholase") activities are associated with the same active site of the phenolase complex molecule, it was of interest...
thicken, become extremely osmiophilic, split lengthwise to form two dense membranes separated by a less dense zone, and finally form a round, double-membraned, type A particle (Fig. 4). The complete formation of these particles will be discussed in more detail elsewhere. The presence of similar particles in the original S-91 melanoma has been briefly noted by Dalton and Felix (4, 5). The particles measure approximately 73–94 nm in diameter, with the inner membrane being slightly thicker and more osmiophilic than the outer one. On occasion the particles may be found free within the cytoplasm or, rarely, extracellularly. Those found outside the cell possess a central dense nucleoid and are probably type B particles.

A particle (Fig. 4). The complete formation of these particles will be discussed in more detail elsewhere. The presence of similar particles in the original S-91 melanoma has been briefly noted by Dalton and Felix (4, 5). The particles measure approximately 73–94 nm in diameter, with the inner membrane being slightly thicker and more osmiophilic than the outer one. On occasion the particles may be found free within the cytoplasm or, rarely, extracellularly. Those found outside the cell possess a central dense nucleoid and are probably type B particles.

**DISCUSSION**

Fitzpatrick et al. (7) and Fitzpatrick and Kukita (8) have classified tyrosinase (phenolase complex) activity into three types:

A. **tyrosinase (+), dopa oxidase (+).** Occurs in the presence of relatively high concentration of the phenolase complex.

B. **tyrosinase (−), dopa oxidase (+).** Occurs in the presence of lower concentrations or partial inhibition of the phenolase complex.

C. **tyrosinase (−), dopa oxidase (−).** Occurs when the phenolase complex is completely inhibited or absent.

On first examination the S-91 variant seemed to fall under category B. However, careful investigation demonstrated the concomitant presence of tyrosinase activity. If, as seems likely, this is also the case in other systems, it may obviate the necessity for a classification B. The presence of tyrosinase activity in intact tissue (as contrasted with its absence in an extract prepared by extremely mild procedures) should do much to negate the recurrent assertions that dopa oxidase activity occurs in a cell or tissue in the absence of tyrosinase activity (see, e.g. [39] for a more recent resuscitation of this original concept of Bloch). The present results appear to add confirmation to the data and hypotheses brought forth by others (3, 20, 25, 27, 31, 32, 34) that tyrosinase and dopa oxidase are a single enzyme entity with two different functions.

It has been shown above that dopa oxidase activity is competitively inhibited by tyrosine. This appears to be the first time such findings have been made with mammalian phenolase complex preparations, although with mushroom tyrosinase Osaki (35) found that tyrosine competitively inhibited dopa oxidation. Kendal (25), using mushroom tyrosinase preparations, could not demonstrate this phenomenon but did find competitive inhibition of catechol oxidation by phenol.

The present data show that even though tyrosinase activity has been inactivated in the process of preparing the tissue extracts and only dopa oxidase activity remains, tyrosine and dopa still compete for the same enzyme site. These findings may indicate that the active sites on the enzyme surface are the same for both substrates or that they are in close approximation; however, the data by no means rule out the possibility that two more distant sites on the phenolase complex molecule are involved (s_t, the tyrosine site; s_d, the dopa site) and that s_t has an affinity for tyrosine.

Their ultrastructural appearance and biochemical characteristics place the melanocytes of this tumor variant between those of the albino mammal or amelanotic melanoma and those of the pigmented animal or melanotic melanoma. In the former, only melanosomes (immature melanin particles) are present, whereas the latter are characterized by the presence of mature melanin granules (2, 33, 43, 44). The variant here studied is characterized by a preponderance of melanosomes and only relatively few mature melanin granules. We believe this to be a genetically determined effect; the variant melanocytes may have been present in the original melanoma or may have arisen by mutation during passage in tissue culture, where selection for this type may have taken place. The genetic effect seems to be such that an unstable enzyme is produced and localization of the enzyme on the melanosome structure is retarded.

The present tumor exhibits a low phenolase complex activity. However, there is considerable variability in activity from tumor to tumor. This variability, apparent both by inspection of the gross pigmentation of the tumor and from assays for either tyrosinase or dopa oxidase, could be directly correlated with the presence or absence of an inhibitor of melanogenesis. Presence of the inhibitor can be demonstrated directly in experiments in which extracts from enzymatically active tumors and from tumors containing the inhibitor are mixed. Presence or absence of the inhibitor seems to be in part genetically determined, as selection for or against this character could be achieved. Activity of the inhibitor diminishes with increasing age of the tumor. In tissue extracts the inhibitor is inactivated during storage at low temperatures. Similar observations have already been made (17, 21) in experiments with tyrosinase from Neurospora crassa, where it was found that storage of the mycelium at 0°C or dry ice temperatures increased tyrosinase activity considerably and induced activity in initially inactive preparations. A similar phenomenon has been observed (13) in dopa oxidase preparations from guinea pig skin.

Heat-labile, nondialyzable inhibitors of enzymatic tyrosinase and dopa oxidation and of dopa autoxidation have been described in mouse melanoma extracts by Riley et al. (37, 38). The presence of inhibitors of melanogenesis in such diverse biological material as molds, frog skin, melanomas, as well as normal mammalian tissues, has been discussed in detail (19). An interesting contribution to this subject has been made recently by Karkhanis and Frieden (23, 24), who isolated an inhibitor protein associated with mushroom tyrosinase and have shown that it can be converted to active tyrosinase on addition of cupric ion.

It is apparent from the present findings that the inhibitor is physiologically active and that its presence or absence to a large extent determines the actual melanization of the tumor. Whether the present inhibitor of melanogenesis is a "proenzyme" similar to that found by a number of authors (for discussion, see [12]) or the apoenzyme inhibitor of Karkhanis and Frieden (23, 24) is a question of consid-
erable interest. Certainly the presence of such inhibitors and their eventual removal or inactivation have implications for such problems as the transformation of melanotic to amelanotic melanomas, the genetic control of melanogenesis in pigment cells, and processes of melan differentiation.

ACKNOWLEDGMENTS

Dr. Robert F. Acker, Microbiological Associates, Inc., Bethesda, Maryland, kindly supplied the tumor material used. The authors are also grateful to Dr. Erhard Haus for his histological examination of the tumor, to Dr. L. W. Fowiks for much helpful advice, and to Mrs. Judith Strong and Miss Bernice Uittenbogaard for valuable technical assistance.

REFERENCES

Fig. 1.—Electron microscopy of the S-91 melanoma variant. The cell membranes (cm) are relatively straight and closely approximate one another. Desmosomes (d) are present and are not well developed. Melanosomes (me) in several stages of maturity are present. N, nucleus; M, mitochondrion.

Type A particles (arrow) are present at the upper left. The endoplasmic reticulum (er) is well developed. Numerous, faintly osmiophilic sacs (v) are present and are continuous with the endoplasmic reticulum. Dense membranes (m) are present in the region of these newly formed vesicles. Age of the tumor, 27 days. X 27,590.

Fig. 2.—Ultrastructural aspects of melanosomes and melanin granules of the S-91 melanoma variant. Numerous melanosomes are visible in this micrograph. A number of partially melanized particles are also seen. The striated membrane pattern of the melanosome is well demonstrated at this high magnification. Age of the tumor, 27 days. X 91,830.
Fig. 3.—Formation of type A particles in the S-91 melanoma variant. This micrograph shows the continuity between the newly formed vesicles (v) and the more mature endoplasmic reticulum (er). The walls of the new vesicles have not thickened and become densely osmiophilic. M, mitochondrion. X 41,380.
Fig. 4.—Formation of type A particles in the S-91 melanoma variant. Following their formation the thin-walled vesicles (v) thicken and become very osmiophilic (o). Some mature type A, doughnut-shaped particles are also present. × 39,880.
An Enzymatic and Electron Microscopic Characterization of a Variant of the Cloudman S-91 Melanoma

Herbert M. Hirsch and Alvin S. Zelickson

Cancer Res 1964;24:1137-1153.

Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/24/7/1137

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.