Biochemistry of Malignant Melanoma

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ABSTRACT

The biochemistry of malignant melanoma is reviewed. The biosynthesis of melanin from tyrosine is described and the role of tyrosinase and other enzymes in melanoma considered. Detailed methods for the assay of free catechols, their metabolites and urinary indole melanogens are included. Normal values for these constituents and their significance in the evaluation of melanoma patients are discussed.

The biochemistry of malignant melanoma is essentially that of accelerated or uncontrolled activity of enzymes involved in the biosynthetic and/or degradative pathways of tyrosine metabolism and the subsequent formation of supranormal amounts of melanogens and the black pigment, melanin (figure 1). However, the role of the clinical biochemistry laboratory in the diagnosis and clinical management of malignant melanoma has not been an important one. Relatively little attention has been paid to examination of the intermediates in melanin synthesis and quantitative assays of melanogens in urine have not been particularly useful in evaluating the disease.

Melanin Biosynthesis

Melanin biosynthesis occurs in the Golgi region of melanocytes, cells which are derived from melanoblasts and are embryologically of neural crest origin. The melanocytes contain tyrosinase which initiates the formation of melanin from tyrosine by progressive layering of melanin polymer on the surface of a protein containing the enzyme activity. As the melanin deposits become more dense, the tyrosinase is gradually inactivated. Melanosomes, the particles containing the layers of melanin, vary in diameter from 0.3 mm to 1.0 mm and as much as 30 percent of their weight may be melanin. In skin pigmentation, melanosomes are mobilized and directed by the pituitary melanocyte stimulating hormone (MSH). The role of MSH in melanoma is not understood. Indirect evidence for a pituitary role in melanoma is the observation that both adrenocorticotropic hormone (ACTH) and MSH have a stimulating effect on the growth of human malignant melanoma cells in culture.11,15

In the conversion of tyrosine to dihydroxyphenylalanine DOPA quinone, tyrosinase is involved in a cyclic, oxygen mediated electron transfer. DOPA serves as an intermediate and a necessary primer. The active form of the enzyme requires intramolecular copper in the cuprous state. Tyrosinase purified from mouse melanoma can catalyze the oxidation of tyrosine, but not tyrosine derivate in which the free amino or phenol groups are substituted. The enzyme is inhibited competitively by phenylalanine, fluoride, chloride, acetate, oxalate and irreversibly by mercury, gold or silver. Acrylamide gel electrophoresis se-
Figure 1. Metabolism of tyrosine.
parates tyrosinase from melanoma tissue into three distinct areas of activity. The middle band is the dominant form and can be separated from the others by precipitation with 50 to 70 percent saturated ammonium sulfate. Despite the established role of tyrosinase in melanization, the extent of melanin formation is not related directly to the enzyme activity. A soluble, dialyzable, heat stable protein inhibitor with a molecular weight of less than 10,000 has been isolated from mouse melanoma tissue. Storage of mouse melanoma tissue at —25° for two years increased the tyrosinase activity two to four fold, presumably owing to loss of a naturally occurring inhibitor.

Tyrosinase is present in relatively high concentrations in human melanoma tissue. In a study of tyrosinase prepared from normal skin and metastatic melanoma from the same negro patient, 46 to 95 units per mg were found in the skin and 17,000 to 19,500 units per mg in the melanoma. In skin, all activity was observed in the particulate fraction, but in melanoma about 75 percent was in the particulate fraction and the remainder in the supernatant obtained by centrifugation at 144,000 x g for 40 minutes at 0 to 6°.

Although tyrosinase is considered the major enzyme in melanin formation, melanocytes also contain hemeproteins with peroxidase activity. Okun and his associates have presented evidence that peroxidase mediates the initial hydroxylation of tyrosine. According to this proposal, tyrosinase is strictly a "DOPA oxidase" which catalyzes the conversion of the peroxidase mediated DOPA to DOPA quinone. Support for the role of peroxidase in melanogenesis is the fact that oxidation of tyrosine to melanin is completely suppressed by added catalase which competitively depletes hydrogen peroxide in the system.

The only enzymatic conversions in the formation of melanin are the tyrosinase and possibly the peroxidase steps. The remaining compounds are formed non-enzymatically. DOPA quinone is unstable and undergoes immediate non-enzymatic intramolecular rearrangement to form 5,6-dihydroxyindole-2-carboxylic acid which then undergoes rearrangement to yield 5,6 dihydroxyindole. The non-enzymatic reactions appear to be pH dependent (pH 5.6). Melanin itself results from spontaneous polymerization of the 5,6 dihydroxyindole molecules through 3,7 linkages with the release of CO₂. The precise structure of melanin has been difficult to define. This is because of the insolubility of melanin and the fact that intracellularly melanin is tightly complexed with protein. If the protein is removed by acid or enzymatic hydrolysis, there is no assurance that the procedure has not altered the structure of the melanin. The protein is thought to have a molecular weight of 20,000 and to contain a large proportion of cystine-cysteine, methionine, arginine, histidine, tyrosine, tryptophane and 0.2 percent to 0.8 percent DOPA.

Although melanin formation is a biochemical phenomenon characteristic of melanoma, it must be kept in mind that hyperpigmentation occurs in other situations. Lerner has observed that pigmentation may be related to (1) genetic disorders such as pigmented nevi or intestinal polyposis, (2) physical factors such as ionizing radiations or heat, (3) chemical factors as seen in heavy metal intoxication, (4) endocrine factors (Addison's Disease, or hyperthyroidism) and (5) in nutritional deficiencies such as pellagra.

The assay of tyrosinase has not been useful as an aid in the diagnosis or staging of melanoma or in following the course of the disease and the enzyme is only detectable in serum in patients with advanced disease. However, increased tyrosinase activity and concomitant DOPA formation results in the formation of many metabolites which may be found in the blood or excreted in the urine in abnormal amounts by patients with melanoma (figure 1). These intermediates can be grouped into four categories; indole
or phenol melanogens, free catechols, and melanin. In addition, a cysteine derivate of DOPA has been described (figure 1).

**Indole Melanogens**

Indole melanogens are melanin precursors excreted into the urine as colorless compounds. Occasionally, the directly voided urine of a melanoma patient is dark in color and obviously contains melanin. In these cases, oxidation and polymerization have occurred in the bladder. Indole melanogens can be water soluble esters (glucuronide or sulfate) or non-esterified compounds which are soluble in ether or ethyl acetate. These compounds include 5-hydroxy-6-methoxy indole-5-monoglucuronide, 5-methoxy-6-hydroxyindole-6-monoglucuronide, 5,6-dihydroxyindole-5-monoglucuronide, 5,6-dihydroxyindole-6-monoglucuronide, 5,6-dihydroxyindole-5-monosulfate, 5,6-dihydroxyindole-6-monosulfate, 5-hydroxy-6-methoxy-2-carboxylic acid, 5-methoxy-6-hydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole.

The metabolites with an unsubstituted pyrrol ring (all of the previously mentioned except the carboxylic acid derivatives of 5,6 dihydroxyindole) will react with an alkaline aqueous solution of nitroferricyanide to yield a violet colored complex which when acidified becomes blue or blue-green. This reaction has been adopted into the widely used Thormahler test and is highly specific for indolic melanogens with any phenolic grouping but an unsubstituted pyrrol nucleus. Both unconjugated indoles and glucuronide or sulfate conjugates will react. The test is performed in the following manner:

1. The patient is maintained free of salicylate and barbiturate drugs for three days prior to the urine collection. Since melanuria may be either continuous or episodic, it is important to obtain urine during a 24-hour period and over several days. Canned fruits are avoided because certain preservatives used in canning may interfere. In addition, irradiation or vitamin C administration may cause melanuria. If the urine is also to be used for catechol determination, the patient should avoid foods containing vanilla or cough preparations containing methyl guaicol. It has been recommended that concentrated urine is the most appropriate sample. Therefore, diuretics should be avoided and the patient should be maintained without liquids during the 12 to 18 hours prior to the start of the collection.

2. Twenty-four hour collections must contain 1 g sodium sulfate and be maintained in an oxygen-free state by layering with toluene.

3. Four ml aliquots of urine are pipeted into test tubes. To one aliquot, 0.1 ml of water is added. To each of the other aliquots is added precisely 0.1 ml aliquot of indole standard diluted to permit construction of a standard curve ranging from 2 μg to 20 μg indole per tube. The stock indole standard (200 μg per ml) is prepared by dissolving 20 mg of pure indole in 2 ml of 95 percent ethanol and then diluting to 100 ml with water. A reagent blank contains 4.1 ml of water.

4. To all tubes are added with mixing, 0.5 ml of 2 percent (w/v) fresh sodium nitroprusside and then 2 ml of 10 percent (w/v) sodium hydroxide.

5. After exactly two minutes (accurately timed and constant for each tube), 0.2 ml of glacial acetic acid is added and the tubes are vigorously mixed.

6. The absorbancies of the resulting blue colored solutions are read within 20 minutes at 620 nm using the reagent blank to adjust the spectrophotometer. The molecular extraction coefficient of purified indole is 6.4 × 10^13.

7. The concentration of indole in the four ml urine aliquot is determined from the standard curve. The reaction is linear through concentrations of 5 to 100 μg of indole in the final volume.

Other procedures have utilized different
volumes and in one method absorbancies are recorded through a spectrum between 400 nm to 700 nm. Melanin is present if the absorbancy at 625 exceeds that at 525 nm. Plasma melanin may be determined by placing one ml of heparinized plasma on a Sephadex G-25 column, eluting with water and treating the eluates as described for urine.

In 10 normal persons the urine concentration was reported to be 4.5 ± 1.8 μg per ml and in patients with melanoma, 30 to 50 μg per ml with observed values up to 120 μg per ml. In general, a value of 5 μg per ml or 6 mg or less in a 24-hour specimen has been considered a normal excretion. In random specimens or incomplete 24 hour specimens it has been suggested that the normal-abnormal cutoff be 10 μg per ml or 8 mg per 24 hours.

Another procedure for melanin determinations is the ferric chloride technique of Jaksch-Pollak. This is a non-specific reaction in which 0.2 ml of 5 percent (w/v) ferric chloride in 1 N HCl is added to 10 ml fresh urine. After centrifugation, a positive test is indicated by a gray to gray-black precipitate and on occasion, a dark gray supernate. Brown and other colors are negative reactions.

Many workers feel that melanuria is not observed when small tumors are present and that an observation of melanuria in itself is not a valuable diagnostic test. In a study relating the clinical stage of melanoma to the presence of melanuria, it was observed that 9 of 11 patients with primary tumor but no evidence of metastases (Stage I), did not excrete indole melanogens in their urine and two other patients demonstrated equivocal amounts. Only one of 10 patients with regional lymph node metastases (Stage II) and one of six patients with distant metastases without hepatomegaly (Stage III) exhibited positive melanuria. Elevations were observed in all of five patients with distant metastases and liver involvement (Stage IV). In one patient with an enlarged liver who excreted an abnormal amount of melanogens (30 mg per g creatinine), successful treatment with cytotoxic drugs resulted in a decrease in liver size and a rapid fall in the urinary excretion of melanogens. There was a return toward elevated melanin values during a terminal exacerbation of the disease. In another study only 56 of 263 patients with melanoma excreted abnormal amounts of melanogens (> 10 μg per ml). Elevations were observed in 5 percent of patients with Stage I disease, 20 percent of those with Stage II and 80 percent of the patients with liver disease (Stage III). A review of the literature (March 1972) by Duchon and Matois revealed elevations of urinary melanogens in 110 of 457 (24 percent) of patients. Specific analysis of individual metabolites does not improve the extent of elevations. When 5-hydroxy-6-methoxy- and 6-methoxy-5-hydroxyindole-2-carboxylic acids were measured, abnormal concentrations were found in 21 of 95 patients.

Free Catechols and Their Metabolites

The free catechols, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylethylamine (Dopamine) and 3,4 dihydroxyphenylacetic acid (DOPAC) (figure 1) are excreted in increased amounts in urine of patients with malignant melanoma and urinary concentrations are reported to be related to increasing tumor mass.

Urine for the determination of these metabolites must be collected at pH 1 to 3 and refrigerated. In the procedure described by Hinterberger and his associates, the collected urine is adjusted to pH 8.0, passed through neutral alumina and eluted with 0.2N H₂SO₄. The eluates from the alumina columns are adjusted to pH 6.5 and, after the addition of 50 mg of ascorbic acid, are placed on DOWEX 50-X8 resin columns. The DOPAC passes through the column, the adsorbed DOPA is eluted with 20 ml 0.1 M sodium acetate buffer, pH 6.0, noradrenalin
and adrenalin with 10 ml N HCl and then Dopamine with 10 ml 2 N HCl. The individual compounds are determined fluorometrically. Numerous methods including automated procedures have been described for the assay of DOPA in urine. The technique proposed by Turler and Kaser is as follows:

1. Two 50 ml aliquots of acidified urine are taken and to each is added 1 ml of a solution containing 0.2 M EDTA and 1 percent sodium metabisulfite. One urine serves as an internal control and to it is added one ml of a standard containing 4 µg of DOPA. One ml of water is added to the assay urine. The pH of both aliquots is adjusted to pH 8.7 with 2.5 M K₂CO₃, maintained at 0° and centrifuged in the cold for five minutes at 6000 x g.

2. Two g of aluminum oxide (Woelms neutral alumina is washed six successive times with 500 ml portions of 1 N acetic acid, 10 times with 500 ml of water or until the pH of the wash water is neutral and the sediment is then dried for four hours at 110°) are added to the urine.

3. The urine-alumina mixture is stirred vigorously first for five minutes while the pH is kept at 3.7 with an automatic titrator, then for 10 additional minutes without pH adjustment. The mixture is filtered through a sintered glass filter (solution A).

4. The precipitate is washed with 5 ml 1 percent KHCO₃, pH 8.7 (solution B).

5. Solution A and B are combined and may be used for the assay of 3-methoxytyramine, normetanephrine and metanephrine.

6. The alumina is then washed with a solution of 18.5 ml of 0.5 N acetic acid, one ml 1 percent sodium metabisulfite and 0.5 ml 0.2 M EDTA solution (solution C).

7. Solution C is adjusted to pH 5.5 with about 2 ml of 0.5 M phosphate buffer containing 2.5 M K₂CO₃ and placed on a four mm interior diameter capillary column containing 600 mg of the sodium form of AG 50W X12 - 400 mesh resin suspended first in 0.5 M phosphate buffer, pH 5.5, on a sintered glass filter and then washed with 10 ml of 0.067 M phosphate buffer, pH 5.5. The solution is forced through the capillary at a rate of 0.7 to 0.8 ml per minute and the sediment washed at the same flow rate with 20 ml of 0.065 M phosphate buffer, pH 5.5. The washing and the column treated solution C are combined and used for the DOPA assay (solution D).

8. Solution D containing the DOPA is concentrated by adsorption on one g of alumina, then adjusting the pH of the mixture to 8.6 with K₂CO₃, filtering, washing the sediment twice with five ml portions of 1 percent KHCO₃, pH 6.8 and finally eluting the DOPA with 8 ml of 0.5 N acetic acid followed by 2 ml 1 N HCl (solution E). To solution E are added one ml of 0.5 M citric acid and one ml 0.5 M boric acid, the pH is adjusted to 5.5 with 2.5 M K₂CO₃ and the final volume brought to 16 ml (solution F).

9. To three ml aliquots of solution F from the unknown urine sample, the internal standard and the reagent blank, the following are added in sequence:

**Assay Sample.** Precisely 0.2 ml water and 0.05 ml of 0.01 M iodine solution are added. After five minutes, 0.5 ml of freshly prepared 0.2 M sodium thiosulphite in 4.5 M sodium hydroxide is added and after five additional minutes, 1.0 ml 5 N acetic acid is added.

**Internal Standard.** Exactly 0.2 ml of DOPA solution is added (4 µg per ml) instead of the water and then the other reagents are added in the same sequence as the unknown urine.

**External Standard.** The water is added first to the reagent blank. Following that are added the alkaline sulfitreagent, the acetic acid and, finally, the iodine solution.

10. All of the tubes are allowed to stand for 30 minutes at 60° and the fluorescence measured with an activation frequency of 333 nm and an emission frequency of 388 nm.

11. The µg of DOPA is calculated by
free catechols by melanoma patients. As can be seen, the excretion is related to the extent of the disease.

In table II are shown the further results of this study in which the individual free catechols were determined. Two of four patients with Stage I disease excreted moderately increased amounts of DOPA. Slight elevations of Dopamine and DOPAC were observed in one of these patients. DOPA excretions were elevated from four to 65 times the normal mean in all patients with Stage II disease, 13 to 188 fold in Stage III, and three to 1070 fold in Stage IV. Dopamine and DOPAC were elevated in fewer patients, dopamine in 6 of 10 patients and DOPAC in 4 of 9 patients with Stage IV disease. It cannot be stated that DOPA assays alone are sufficient to evaluate the disease. In a patient with normal excretions of DOPA (21 µg per ml) and DOPAC (0 µg per ml), the Dopamine was excreted at a level of 348 µg per ml. In another study (table III) the specificity of urinary DOPA assays was improved by expressing the values as µg per mg creatinine. Blood DOPA, not detectable in normal plasma, is found in the plasma of melanoma patients, but its presence in blood is not related to urinary catechols.

DOPA is excreted in excessive amounts in about 63 percent of melanoma patients. Elevations of DOPAC and Dopamine are only observed in about 20 percent of such patients. Duchon and Matois, in a review of literature, found that free catechols were

<table>
<thead>
<tr>
<th>Disease Stage</th>
<th>Number of Patients</th>
<th>Total Catechols (µg per 24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Controls</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>0</td>
<td>19</td>
<td>122</td>
</tr>
<tr>
<td>I</td>
<td>19</td>
<td>146</td>
</tr>
<tr>
<td>II</td>
<td>25</td>
<td>238</td>
</tr>
<tr>
<td>III</td>
<td>23</td>
<td>316</td>
</tr>
<tr>
<td>IV</td>
<td>27</td>
<td>1,347</td>
</tr>
</tbody>
</table>

**TABLE II**

Excretion of Free Catechols by Melanoma Patients

<table>
<thead>
<tr>
<th>Disease Stage</th>
<th>Number of Patients</th>
<th>DOPA Mean (range)</th>
<th>Metabolite (µg per 24 hours)</th>
<th>DOPAC Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DOPA Mean (range)</td>
<td>Dopamine Mean (range)</td>
<td>DopAC Mean (range)</td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>6 (0-24)</td>
<td>78 (42-130)</td>
<td>600 (0-750)</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>7 (0-13)</td>
<td>50 (15-45)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>41 (9-93)</td>
<td>127 (93-167)</td>
<td>1213 (738-2100)</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>170 (59-391)</td>
<td>78 (40-118)</td>
<td>304 (0-569)</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>392 (79-1126)</td>
<td>63 (10-157)</td>
<td>900 (285-2013)</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>1076 (21-6420)</td>
<td>275 (0-1132)</td>
<td>4889 (0-27,795)</td>
</tr>
</tbody>
</table>
TABLE III

Urinary DOPA in Patients with Melanoma

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of Patients</th>
<th>DOPA (mg per g Creatinine) (Mean, Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8</td>
<td>19 (8-30)</td>
</tr>
<tr>
<td>Melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amelanotic</td>
<td>2</td>
<td>30 (29-31)</td>
</tr>
<tr>
<td>Partially amelanotic</td>
<td>4</td>
<td>58 (45-74)</td>
</tr>
<tr>
<td>Local metastases</td>
<td>14</td>
<td>60 (26-1300)</td>
</tr>
<tr>
<td>Distant metastases</td>
<td>19</td>
<td>520 (19-2490)</td>
</tr>
</tbody>
</table>

Elevated in 28 of 45 patients, DOPA without regard for stage in 26 of 53 patients and DOPAC in 23 of 99 patients. The degradation product of Dopamine, homovanillic acid (HVA), was excreted in abnormal amounts in only 34 of 158 patients. In a similar study, DOPA was abnormal in 12 of 13 patients and HVA in only seven of the subjects.

In a simple procedure described for the assay of HVA, five ml of urine are diluted with an equal volume of water saturated with solid NaCl, acidified to pH 1 or less with 6 N HCl and extracted with 10 ml of methylene chloride. The solvent phase is dried with Na₂SO₄ and evaporated to dryness at room temperature. The residue is dissolved in one ml of alkaline water, neutralized and chromatographed on an AG 50W-X4 cation exchange resin and eluted with 0.3 percent NaCl. The eluate is made alkaline with NaOH and oxidized with potassium ferricyanide. After destruction of the excess oxidant with cysteine, the HVA is determined fluorometrically with an activation frequency of 300 nm and an emission frequency at 430 nm.

Cysteinyl DOPA (figure 1) has been observed in urine of patients with melanoma. In the assay, the compound is extracted from urine with perchloric acid and adsorbed to alumina. The material is eluted and separated from the other catechols, oxidized with iodine and sodium sulfite and then determined fluorometrically by excitation at 325 nm and emission at 405 nm. At these frequencies, other catechols do not interfere. In urine of 10 normal persons and two patients with excised melanoma, the 5-S-cysteinyl DOPA ranged from 0.0 to 0.04 μg per ml. In four patients with metastatic melanoma, the excretions ranged between 0.32 to 1.40 μg per ml.

Hemopexin

Other than the possible assay of blood DOPA or "DOPA oxidase" serum or plasma determinations have not been useful in monitoring melanoma. Plasma CEA has been measured in melanoma patients and in our study elevations were observed in only nine of 58 patients. Hemopexin has been reported to be present in abnormal concentrations in plasma of melanoma patients. Hemopexin is a plasma glycoprotein with a molecular weight of about 80,000. It contains 9.0 percent hexose, 7.1 percent acetyl hexosamine, 5.8 percent sialic acid and 0.4 percent fucose. Synonyms for hemopexin include heme binding β-globulin, serumucoid, cytochromophilin or β1-haptoglobin. Hemopexin assays have been performed by radial immunodiffusion technique. A mean level in 70 normal individuals was found to be 98 ± 10 units with a range between 60 to 100 units. Similar values were found in 20 patients with tumors other than melanoma. Modestly elevated values were observed in patients with slowly-evolving melanomas, but markedly elevated values were observed in patients with rapidly progressing tumor. In 100 patients with melanoma the value ranged from 40 to 400 units with a mean of 124 units. In patients with no change in clinical status in the three months before and after the assay (slowly evolving melanomas) the values ranged between 40 to 100 units with a mean of 77.4 units. However, in rapidly progressing disease the levels ranged between 100 to 260 units with a mean value of 170.5 units. All but three of these patients had values higher than the mean value observed in the patients.
with slowly evolving disease. The data was not related to stage of disease. The significance of hemopexin in the biochemistry of melanoma has not been explained. Its measurement may prove useful in establishing the rapidity of growth of melanoma.

Conclusions

Biochemistry procedures do not permit early diagnosis of melanoma. Normal values of most of the tyrosine metabolites are observed in urine of patients with Stage I and II disease. The present use of biochemistry is in helping to stage the disease and in following its progression. The procedures that are of most promise in this regard are quantitative indole melanogen measurements and the assay of DOPA.

References