On Black Pigment of Gallstones with Special Reference to Difference from Melanins

By

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(Received for publication, December 12, 1964)

Some authors believe that melanins are responsible for occasional black appearance of gallstones and for brown pigmentation of the liver in the case of Dubin-Johnson syndrome. However, it should be doubtful if melanins are really produced in the hepato-biliary system which is known to be devoid of melanophores in the normal condition. To dissolve the problem, black pigments were isolated from black-appearing gallstones and were chemically and physicochemically compared with native and synthetic melanins. Although the gallstone pigments resembled melanins in general properties, a definite difference could be pointed out between both kinds of pigments in the infrared absorption spectra, indicating a probable difference in the structure of these substances.

Although the presence of gallstones that appear black on the surface and transections has long been known to surgeons, no definitive information is yet available as to the origin and composition of these gallstones. Of such black gallstones, those which are occasionally encountered in the case of hemolytic jaundice and are characterized by metallic luster have been customarily referred to as pure pigment stones. But the nomenclature may not be reasonable since such gallstones contain considerable quantities of calcium and other metals besides bilirubin. Recently, Miyake and his collaborators have claimed that the pigments in some black gallstones fall under the category of melanin. However, it may be questionable if such a large amount of melanin pigments as to constitute a gallstone can be produced in the hepato-biliary system which is believed not to contain melanins in the normal condition. At any event, elucidation of the chemical nature and mechanism of formation of the black pigments would afford a clue to the pathogenesis of this type of gallstones.

To approach the problem from the basic standpoint, the black pigments of gallstone were chemically and physico-chemically compared with native and synthetic melanin pigments and further with black pigments derived from natural and synthetic calcium bilirubinate stones.
MATERIALS AND METHODS

1. Preparation of melanin pigments

Specimens of native melanins were isolated from the human black hair, ink sac of Sepia, choroid of the ox eye and human melanoma according to the techniques appearing in literature. Synthetic melanin pigments were prepared in the usual manner from L-tyrosine and from dl-3,4-dihydroxyphenylalanine (dopa).

2. Extraction of black pigments of gallstone

Two surgical specimens of black gallstones, one (No. 1) from a case of hemolytic jaundice and the other (No. 2) from a patient without hemolytic jaundice, served as the materials for extraction of black pigments. The extraction procedure is summarized in Table I. The dried specimen of gallstone was powdered in a mortar and was further dried in a sulfuric acid desiccator under reduced pressure. A 0.5 to 1.0 g portion of the dry powder (I) was heated at 85°C for 48 hours with 20% hydrochloric acid, and the resulting black sediment was isolated by filtration and washed thoroughly with distilled water. The sediment (II) was then extracted with ethanol for 24 hours under reflux; the extract (III) was separated, and the residue (IV) was washed with ethanol and then extracted with chloroform for 24 hours by refluxing. The extract (V) was isolated and the residue (VI),

<table>
<thead>
<tr>
<th>Table I. Scheme for Extraction of Black Pigments from Gallstone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powdered gallstone (I)</td>
</tr>
<tr>
<td>Treated with 20% HCl at 85°C for 48 hrs.</td>
</tr>
<tr>
<td>Filtered</td>
</tr>
<tr>
<td>Filtrate Residue (II)</td>
</tr>
<tr>
<td>Refluxed in ethanol for 24 hrs.</td>
</tr>
<tr>
<td>Extract (III) Residue (IV)</td>
</tr>
<tr>
<td>Refluxed in chloroform for 24 hrs.</td>
</tr>
<tr>
<td>Extract (V) Residue (VI)</td>
</tr>
<tr>
<td>Extracted with 5% NH₄OH</td>
</tr>
<tr>
<td>Extract (VII) Residue (VIII)</td>
</tr>
<tr>
<td>The above extraction procedure is repeated.</td>
</tr>
<tr>
<td>Extracts III', V' &amp; VII'</td>
</tr>
<tr>
<td>Residue (VIII')</td>
</tr>
</tbody>
</table>
after being washed with chloroform, was extracted with 5% ammonium hydroxide for 24 hours at room temperature. The residue (VIII) was separated from the extract (VII), washed thoroughly with distilled water and again subjected to the above extraction procedure with 20% hydrochloric acid, ethanol, chloroform and 5% ammonium hydroxide. The final residue (VIII’) was washed with ethanol and ether, and dried in a desiccator.

The final residue (VIII’) of the gallstone specimens served as the material of the present investigation, whereas the extracts with ethanol, chloroform and 5% ammonium hydroxide were processed into solid materials for later use. Both ethanol extracts (III and III’) and the chloroform extracts (V and V’) were kept under reduced pressure to remove the solvents, and the resulting solid substances, Fractions III$_R$ and V$_R$, were dried in vacuo in a desiccator. The extracts with aqueous ammonia (VII and VII’), on the other hand, were acidified with 20% hydrochloric acid and allowed to stand at room temperature. The sediment that appeared in this procedure (Fraction VII$_R$) was collected by centrifuging, washed with distilled water and dried in a desiccator under diminished pressure.

3. Methods of comparison

The solubility of melanin and the black pigments (VIII’) was examined with 20% hydrochloric acid, N/10 sodium hydroxide, ethanol, ether, chloroform and acetone. For studying decoloration properties of the samples, the change in color of the samples was observed at intervals in a test solution consisting of 1 ml of N/10 sodium hydroxide and 3 ml of 100 volume hydrogen peroxide and in a control solution without hydrogen peroxide, at room temperature. Ash and nitrogen analyses were performed in the usual manner.

To measure the ultraviolet spectra of the melanin pigments, the samples were dissolved into small portions of N/10 sodium hydroxide and the solutions were diluted with distilled water until the concentration of sodium hydroxide reached about 0.01 N. Since the black pigment (VIII’) of gallstone was hardly soluble in N/10 sodium hydroxide, ultraviolet spectroscopy was run with a filtrate of a mixture of the pigment and N/10 sodium hydroxide, i.e., with a fraction of the pigment that was soluble to the latter solvent. Ultraviolet spectra were measured with Spectrophotometer EPU-2A Typus (Hitachi). Infrared spectra of dry materials were measured by KBr-disk method with Infrared Spectrophotometer EPI-S2 Typus (Hitachi).

RESULTS

1. Properties of melanin pigments

General properties of the native and synthetic melanin pigments examined in this experiment are summarized in Table II. As shown in Fig. 1, the ultraviolet absorption spectra of the pigments showed plain curves without specific absorp-
TABLE II. General Properties of Melanin Preparations

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Method of preparation*</th>
<th>Appearance</th>
<th>Solubility†</th>
<th>Decoloration</th>
<th>Ash</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human hair-melanin a</td>
<td>conc. H₂SO₄</td>
<td>Black brown powder</td>
<td>Hardly soluble</td>
<td>+</td>
<td>+</td>
<td>8.53</td>
</tr>
<tr>
<td>Human hair-melanin b</td>
<td>1N NaOH</td>
<td>Black brown powder</td>
<td>Hardly soluble</td>
<td>+</td>
<td>+</td>
<td>7.36</td>
</tr>
<tr>
<td>Sepia ink-melanin</td>
<td>20% HCl</td>
<td>Black brown powder</td>
<td>Insoluble</td>
<td>+</td>
<td>+</td>
<td>8.55</td>
</tr>
<tr>
<td>Ox choroid-melanin a</td>
<td>conc. H₂SO₄</td>
<td>Black brown powder</td>
<td>Hardly soluble</td>
<td>+</td>
<td>+</td>
<td>8.72</td>
</tr>
<tr>
<td>Ox choroid-melanin b</td>
<td>2N HCl</td>
<td>Black, lustrous</td>
<td>Insoluble</td>
<td>+</td>
<td>+</td>
<td>8.41</td>
</tr>
<tr>
<td>Human melanoma-melanin</td>
<td>20% HCl</td>
<td>Black brown powder</td>
<td>Insoluble</td>
<td>+</td>
<td>+</td>
<td>7.92</td>
</tr>
<tr>
<td>Synthetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine-melanin</td>
<td>Tyrosinase air, pH 8.0</td>
<td>Black granules</td>
<td>Insoluble</td>
<td>+</td>
<td>+</td>
<td>5.37</td>
</tr>
<tr>
<td>Tyrosine-melanin</td>
<td>Tyrosinase O₂, pH 8.0</td>
<td>Black granules</td>
<td>Insoluble</td>
<td>+</td>
<td>+</td>
<td>7.16</td>
</tr>
<tr>
<td>Dopa-melanin</td>
<td>0.01N NaOH air</td>
<td>Black lustrous granules</td>
<td>Insoluble</td>
<td>+</td>
<td>-</td>
<td>7.75</td>
</tr>
</tbody>
</table>

* Solvents used for extraction of native melanins, or methods of oxidative polymerization applied in preparation of synthetic melanins. † Into organic solvents.

Fig. 1. Ultraviolet spectra of melanin preparations. Solvent, 0.01 N NaOH. Type A pattern was seen in ox choroid-melanin b, dopa-melanin and tyrosine-melanin; Type B in ox choroid-melanin a, human hair-melanin a and b; Type C in human melanoma-melanin; and Type D in Sepia ink-melanin.

Absorption maxima, which were classified into Types A, B, C and D from general impression. The infrared spectra were classified into Types A, B, C and D, as shown in Fig. 2, taking into consideration the general trend and site of absorption bands.
Fig. 2. Infrared spectra of melanin preparations. KBr-disk method. Type A pattern was seen in ox choroid-melanin b; Type B in ox choroid-melanin a and human hair-melanin a; Type C in Sepia ink-melanin, human hair-melanin b, human melanoma-melanin and dopa-melanin; and Type D in tyrosine-melanin.

Although the structure of melanin is still unknown, it is generally believed that native melanins are formed from tyrosine by a series of oxidative polymerization that involves tyrosinase. In this relation, probable evidence of stretching vibration of alkane -CH- in the tyrosine-melanin preparation, as seen in the Type D infrared spectrum at 2,850 cm\(^{-1}\) and 2,925 cm\(^{-1}\), might indicate contamination of this preparation with lower intermediate products of the oxidative polymerization. This possibly suggests a defect in the method and/or technique of synthesis of tyrosine-melanin. Consequently, the Type D spectrum will be excluded from further consideration for avoiding confusion. The other types of the infrared spectra were similar to each other in general trend and showed a number of common absorption bands.

The physico-chemical as well as spectroscopic properties of the native and synthetic melanins prepared by the author thus approximately coincided with those described in previous reports.\(^3,6-8,11-14\)

2. Properties of the black pigment of gallstone

Table III shows the general properties of two samples (No. 1 and No. 2) of Fraction VIII', a pigment of black gallstones obtained as the final residue of successive extraction. The two samples resembled each other in the appearance, solubility, decoloration property, etc. The pigments were hardly soluble in N/10 sodium hydroxide, and ultraviolet spectra of the soluble portions gave plain curves without specific absorption maxima. Infrared spectra of the samples are given in Fig. 3. Although the curves did not indicate a characteristic absorption, they were similar to each other in general trend and in the location of absorp-
3. Comparison between the gallstone pigment and melanins

The pigment (Fraction VIII') isolated from black gallstones was black or brownish black in color, just like melanin pigments, but commonly exhibited metallic luster. Dopa-melanin and ox choroid-melanin were also lustrous but the other preparations of melanin were mat in appearance. The gallstone pigment was hardly soluble in N/10 sodium hydroxide, while melanin pigments were usually soluble in the solvent. The gallstone pigment as well as melanins were easily decolorized in the presence of alkaline hydrogen peroxide. Although the former pigment appeared to be decolorized more rapidly than the latter, the difference was not so significant. Nitrogen determination showed 6–8 per cent for the gallstone pigment and 7–8 per cent for melanin preparations, showing not much difference between both kinds of pigments.

In Fig. 4 are shown together the ultraviolet spectra of the gallstone pigment (more exactly, a fraction of the pigment soluble in N/10 sodium hydroxide) and those of the melanin preparations. No appreciable difference was observed
Fig. 4. Comparison of the ultraviolet spectra between melanin preparations (Types A-D, cf. Fig. 1) and Fraction VIII' of the gallstones No. 1 and No. 2.

Fig. 5. Comparison of the infrared spectra between melanin preparations (Types A-C, cf. Fig. 2) and Fraction VIII' of the gallstones No. 1 and No. 2.

among the absorption characteristics in the ultraviolet ranges. As seen in Fig. 5, on the other hand, the infrared absorption spectra of the gallstone pigment were differentiated from those of the melanin preparations (excluding tyrosine-melanin for above-mentioned reason) in the following points: 1) The gallstone pigment always exhibited remarkable absorptions at 2,900 cm\(^{-1}\) and 2,850 cm\(^{-1}\) that probably represented stretching vibration of alkane -CH\(_2\), while melanins did not show such absorptions or only showed a feeble absorption at 2,850 cm\(^{-1}\). 2) The spectra of the gallstone pigment had a broad and strong absorption band in the range of 1,670–1,550 cm\(^{-1}\), while those of melanins had fairly sharp absorption bands at 1,700–1,690 cm\(^{-1}\) and 1,625–1,610 cm\(^{-1}\). The absorption by the gallstone pigment at about 1,670 cm\(^{-1}\) and that of melanins at 1,700–1,690 cm\(^{-1}\) might possibly be
identical in nature, but the absorption by the gallstone pigment at the vicinity of 1,550 cm\(^{-1}\) and that by melanins at 1,625–1,610 cm\(^{-1}\) were apparently specific to respective pigments. 3) Melanins exhibited a medium absorption band in the range 1,300–1,200 cm\(^{-1}\), but no absorption was shown by the gallstone pigment in this range. 4) The gallstone pigment showed considerable absorptions at 1,160 cm\(^{-1}\), 1,090 cm\(^{-1}\) and 960 cm\(^{-1}\), which were not recognized in melanins. From the above results, the structure of the black pigment (Fraction VIII') isolated from gallstones was proved to be quite different from that of melanins.

**DISCUSSION**

Since most of the pigments identified so far in the gallstone are bilirubin or its derivatives, it is quite likely that pigments in unusual black gallstones are also of bilirubin origin. But the presence in the hepato-biliary system of pigments other than bilirubin and its relatives is not completely excluded. In fact, a number of authors have claimed that melanins are responsible for occasional black appearance of gallstones\(^{2}\) and also for brownish pigmentation of the liver tissue in the case of Dubin-Johnson syndrome.\(^{16,17}\) However, it is open to question how melanins are produced in the hepato-biliary system, which is in normal conditions devoid of these pigments, and are incorporated into gallstones.

Melanins are a family of biogenous pigments whose distribution covers a large variety of animals and plants. However, the chemical structure of melanins remains obscure to a large extent so that even identification among tissue melanins, derived melanins, and synthetic melanins is not very simple. In other words, the definition of melanin is still a matter of controversy as may be seen from the fact that Lison\(^{11}\) has tried to define melanin not in chemical terms but from general properties of the pigment including color, solubility, decoloration properties and argentaffine reactions. Under such a situation, it may be very difficult to examine whether a gallstone pigment is identical with or different from a melanin pigment. As the first step of the present study, the author therefore tried to clarify general aspects of melanin using as many native and synthetic preparations as possible.

On the other hand, a black pigment (Fraction VIII') was isolated from black-appearing gallstones and this was compared with the melanin preparations in various physico-chemical and chemical aspects. It was then found that the difference between the black gallstone pigment in question and melanins was not large enough to allow a definitive conclusion so far as color, solubility and decoloration were concerned. The nitrogen content showed comparatively wide variation both for the gallstone pigment and for melanins, and the difference between these pigments was also not conclusive. The variation of the nitrogen content in the melanin preparations is probably due to the facts that pure isolation products of native melanins are difficult to obtain because of their poor solubility into
extracting agents and that synthetic melanins are contaminated to various extent with intermediate products. The variation in the case of the gallstone pigment may also indicate a difficulty in its pure separation.

Ultraviolet spectroscopy of melanin preparations gave the spectra that resembled those reported by previous authors. However, the gallstone pigment was hardly soluble in organic solvents and even in N/10 sodium hydroxide, and the spectra obtained with a solution into the latter solvent did not show any considerable absorption bands. Ultraviolet spectroscopy thus appeared not to be a satisfactory method for comparison of these pigments. It was considered, however, that the very poor solubility of the gallstone pigment in N/10 sodium hydroxide might indicate a difference of this pigment from melanins comparatively soluble in this solvent.

The problem was then examined by infrared spectroscopy which has recently become more and more popularly used in the field of organic chemistry as a very useful tool of analysis. Although the structure of melanin is still unknown as mentioned before, it is generally known that tyrosine, dihydroxyphenylalanine (dopa), histamine, adrenaline and pyrrole are among precursors of melanin pigments. It is also believed that native melanins are formed from tyrosine as follows: In the presence of tyrosinase, tyrosine is oxidized into dopa, and dopa into 5,6-indolquinone, which is further oxidized and polymerized to result in melanins. When infrared spectra of various melanin preparations were investigated from the above viewpoints, the spectrum exhibited by synthetic tyrosine-melanin (Type D spectrum of the author's classification) appeared to show evidence of stretching vibration of alkane -CH-. This was interpreted to suggest contamination of the preparation with intermediate products of the oxidative polymerization of tyrosine. When tyrosine-melanin was therefore excluded from comparison, the other melanin preparations resembled each other in general aspects of the infrared spectra.

Thus comparing the spectra of the gallstone pigment with those of melanins, a number of differences could be pointed out in the location of absorption bands. The most remarkable difference was in the range of wave number from 1,700 to 1,550 cm\(^{-1}\); the gallstone pigment exhibited large and broad absorption bands in the range between 1,670 and 1,550 cm\(^{-1}\), while melanins showed fairly intense absorptions at 1,700–1,690 cm\(^{-1}\) and at 1,625–1,610 cm\(^{-1}\). Other points of perceptible difference included the presence of absorption by alkanes, at about 2,900 cm\(^{-1}\) and 2,850 cm\(^{-1}\), in the spectrum of the gallstone pigment. Conclusively, there was a remarkable difference between the general tendency of infrared spectra of the black pigment (VIII') and melanins.

From these experimental results, it may be concluded that a black pigment in question resembles melanin pigments in general properties but is apparently different from the latter in the chemical structure. The next problem, that is,
whether the pigment belongs to the category of bile pigments, will be discussed in the following paper.

Acknowledgments

The author wishes to express his heartfelt thanks to the late Emeritus Prof. S. Fujise, Department of Chemistry, Faculty of Science, Tohoku University, and Prof. S. Hishida, Department of Chemistry, Nihon University for their constant encouragement and valuable advice.

The expense for this study was defrayed, in part, by the Ministry of Education of Japan through a grant from the Scientific Research Fund 1963, which is gratefully acknowledged. T. Maki.

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