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MELANINS FORMED BY A CULTURE OF Aureobasidium (Pullularia) pullulans ARNAUD (DE BARY), 1910

B. P. Tarasov, N. A. Yurlova, and N. P. Elinov

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The investigation of the structure of melanins is difficult for the following reasons. In the first place, the chemical composition of the substances is complex and heterogeneous. The schemes for the synthesis of the chromophores known from the literature [1] give an idea of the formation of the monomers, but the process of the building up of the polymer and the question of its structure have so far remained unsolved; furthermore, a considerable number of materials are not covered by the existing classification [2]. In the second place, the structural and functional relationships between the components of the biopolymer – its protein moiety and the chromophore – are unknown. In the third place, the use of spectroscopic methods, which give extremely valuable information on structure at the molecular level, encounters fundamental difficulties in the case of multicomponent polymers. In spite of the voluminous literature devoted to the spectroscopic investigation of the melanins [2, 3], there are very few results giving information required for understanding their structure.

The problem of interpretation is the result of the absence of a well-defined structure of the electronic and IR absorption spectra. The lack of contrast of the melanin spectrum is due to the superposition of broad absorption bands characteristic for the fragments of the polymer molecules. The pattern is considerably complicated by intra- and intermolecular interactions. At the same time, the progress achieved recently in spectroscopic methods will, if only partially, enable the difficulties to be overcome and, in combination with other physicochemical methods, will permit the successful use of spectroscopy for the investigation of the structural changes of melanins.

The present paper gives the results of an investigation by physicochemical and spectral methods of the change in the composition and structure of melanins isolated from a culture of Aureobasidium (Pullularia) pullulans, strain 8. We set ourselves the following tasks: to determine the type of chromophore, to follow the change in the quantitative composition of the biopolymer during the growth of the culture, and to determine to what extent the structure and state of the melanin molecules change as the microorganism ages.

The absorption contours in the electron spectrum of alkaline solutions of the melanins before and after hydrolysis (Figs. 1 and 2) are not smooth lines as is stated in the literature [2]. They show inflections due to the fact that the spectrum consists of a number of broad overlapping absorption bands. The positions of the maxima of these bands were determined with the aid of special separating techniques [4]. In this way it was possible to isolate two absorption bands (λ_{max} 280 and 350 nm) in the spectrum of an alkaline solution of brown melanin, and four absorption bands (λ_{max} 280, 350, 490, 620 nm) in the spectrum of black melanin.

The maxima at 280 and 350 nm are common to all the samples investigated. They show the presence of a dicarbonyl fragment in the chromophore molecule [5, 6].

The infrared absorption spectra of the melanins (Fig. 3) contain features relating to the chromophore and to the protein moiety of the polymer, which complicates their assignment. The spectra of the melanins after

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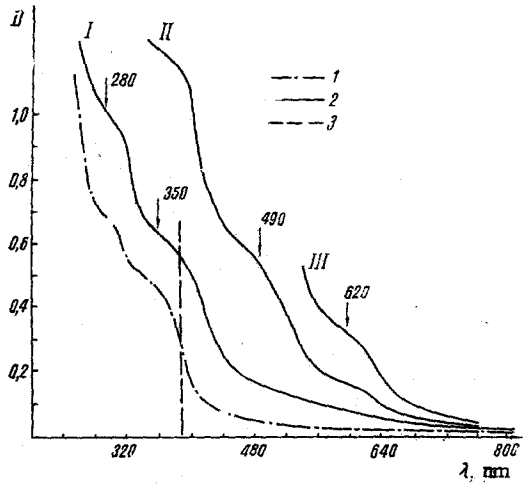

Fig. 1. Visible and UV spectra of the melanins in 0.5 N alkaline solution (samples before hydrolysis): 1) brown; 2) black melanin; 3) passage from a hydrogen lamp to an incandescent lamp; I, II, III) spectra taken in cells with different thicknesses.

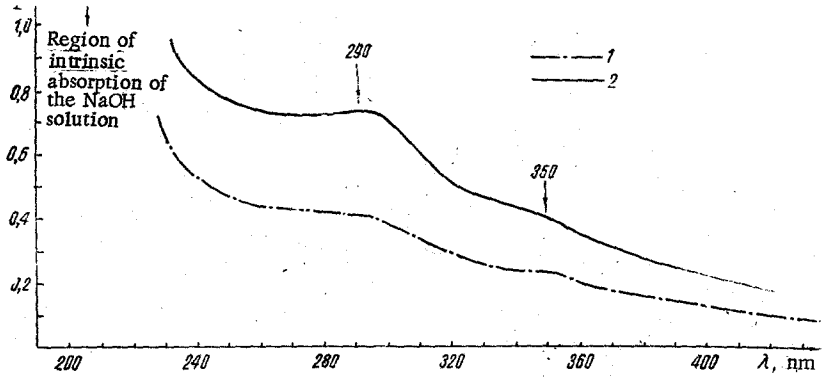

Fig. 2. Absorption spectra of solutions of melanins in 0.5 N NaOH (samples after hydrolysis): 1) brown; 2) black melanin.

acid hydrolysis proved to be simpler. In actual fact, they represent the result of the deduction of the absorption bands of the protein from the spectrum of the initial melanin.

The absorption bands of $\nu_{C=O}$ (sym and asym) at 1635 and 1710 cm^{-1} ($\nu_{C=C}$ makes some contribution to the former), ν_{CH} at 2935 and 2860 cm^{-1} (aromatic and ν_{NH} (aromatic) at 3110 cm^{-1} are the complex characteristics that permit the molecule of the chromophore to be assigned a hydroxyindole structure. An important piece of evidence in favor of this is the positive test for indoles with the Ehrlich reagent. On the whole, all three methods confirm that the samples investigated belong to the melanins of the indole type.

The quantitative and qualitative compositions of the samples were checked by several methods. By means of gel chromatography and paper electrophoresis it was established that the brown melanin before and after hydrolysis has only one fraction, i.e., it is homogeneous (Fig. 4a). Before hydrolysis this fraction includes protein (characteristic green staining of the bands on the electrophoretograms with Bromophenol Blue), after hydrolysis no protein is found. This agrees with the corresponding spectral characteristics.

A different pattern was found in the experiments with the black melanin. Before hydrolysis the pigment included two fractions of which the one with the higher molecular weight contained a polysaccharide. This was confirmed by the staining with fuchshin–sulfurous acid of the band remaining at the starting point on the electrophoretogram, and by a positive reaction with phenol–sulfuric acid of the high-molecular-weight fraction after gel chromatography. It disappeared after hydrolysis, and a single homogeneous – judging from the results of electrophoresis and gel chromatography – fraction of black melanin remained (Fig. 4b).

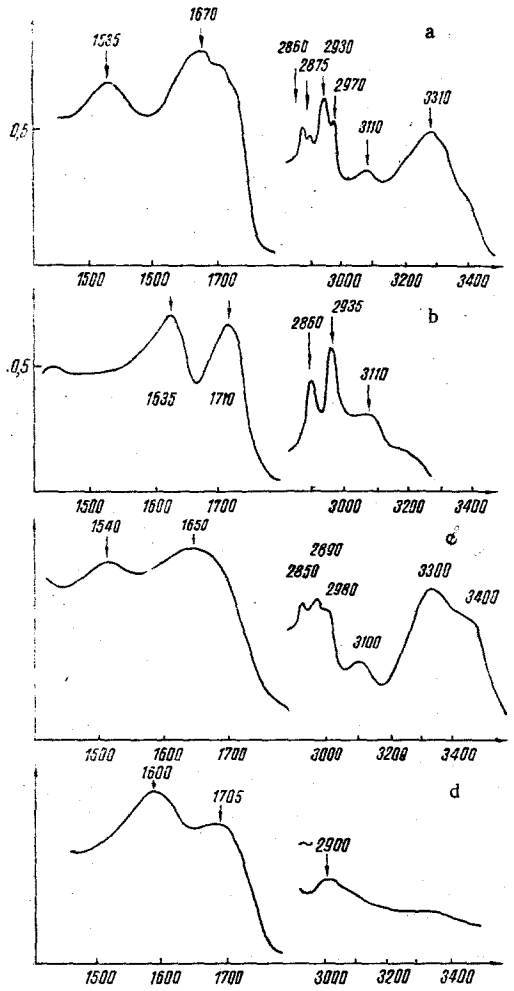

Fig. 3. IR spectra of melanins: a) brown melanin before hydrolysis; b) after hydrolysis; c) black melanin before hydrolysis; d) after hydrolysis.

The determination of the amount of protein components showed that the brown melanin contained 41.6% of protein by weight, and the black melanin only 17.3%.

As we have already mentioned, on careful analysis of the absorption contour of the electronic spectra of the black melanin, two "superfluous" bands were found at 490 and 620 nm, respectively. It is just these that show the characteristic black-brown of the material. These bands are not connected with any impurities but are permanent characteristics of the black pigment [they are present practically unchanged in the high- and low-molecular-weight fractions of the black pigment after gel chromatography (Fig. 5)]. They cannot be assigned to quinone derivatives, since no characteristic decoloration (i.e., a decrease in the intensity of the corresponding peaks) is observed on the addition of alkali.

On the other hand, neither the hydroxyindoles themselves nor their complexes with proteins of polysaccharides can give absorption in this region of the spectrum. Our hypothesis of the photochemical origin of the colored products was not confirmed: prolonged irradiation of solid samples in a quartz celll with a powerful ultraviolet source gave a completely different change in the spectral pattern. It may apparently be assumed that the colored products are stable free radicals formed in the metabolic process of the culture. In favor of this is the fact that similar spectral phenomena have been observed for flavonoproteins [7], and in this case the radical nature of the particles was shown strictly.

Thus, in the process of development of the culture the pigment undergoes a series of quantitative changes the relative protein content decreases and the sample becomes nonhomogeneous – a high-molecular-weight fraction containing a polysaccharide appears. Furthermore, colored products apparently of radical nature arise in the polymer.

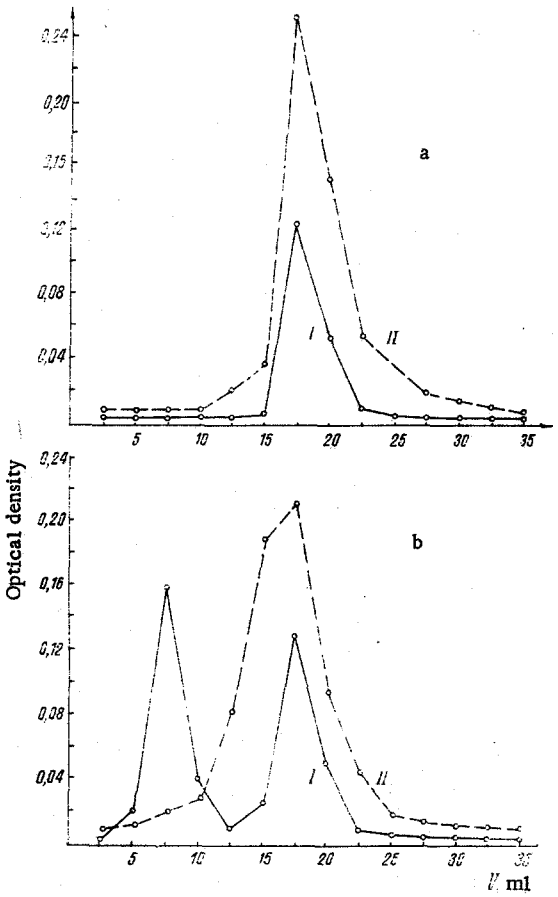

Fig. 4. Gel chromatography on Bio-Gel P-60 of brown (a) and black (b) melanins before (I) and after (II) hydrolysis.

Spectral methods permit features of the structural transformation of the components of the pigment during the development of a culture of Aureobasidium pullulans, strain 8, to be recorded. In the IR spectra of the melanins before hydrolysis (see Fig. 3) it is possible to distinguish the absorption bands of a protein: the bands of $\nu_{C=O}$ at 1670 cm^{-1} (1650 cm^{-1})*, ν_{CH_2} (sym and asym) at 2875 and 2970 cm^{-1}, and ν_{NH} at 3310 cm^{-1} (3300 cm^{-1}) and the amide II band (δ_{NH} deformation vibrations) at 1535 cm^{-1} (1540 cm^{-1}). The slight but clearly detectable differences in the positions of the amide III and $\nu_{C=O}$ bands (with correction for overlapping) give grounds for the assumption that the structure of the protein changes from less ordered (brown melanin) to more ordered (black melanin) [8].

The electronic spectra of aqueous solutions of the pigment obtained after gel chromatography (see Fig. 5) contain an extremely strong band at 195 nm relating to the $\pi - \pi^*$ transition of the C=O groups of a protein. The ratio of the intensities of this band, referred to the weight concentration of the proteins, for the brown melanin and the low- and high-molecular-weight fractions of the black melanin, respectively, is 3.8 : 2 : 1. Such a marked change is probably connected with a change in the state of the carbonyl groups of the protein and, correspondingly, with a decrease in the mean extinction coefficient of the $\pi - \pi^*$ transition. A change in the direction of the vectors of the dipole moments in structural transitions can only increase these differences and, furthermore, the corresponding changes amount to 70% of the value of ε for 100% helix–coil transition [8]. One of the most probable causes of this change is the cleavage of the hydrogen bonds between water molecules forming the hydrate shell and the C=O groups of the protein. It is natural that the process has a collective nature, i.e., the ensemble of C=O...HOH H-bonds is replaced by an inadequate number of hydrogen bonds between the fragments of the protein molecule, whereupon the average extinction coefficient of the sample falls [4].

*The corresponding figures for black melanin are given in parentheses. In view of the overlapping of the bands of the chromophore and of the protein the position of $\nu_{C=O}$ is given for the center of gravity of the combined band.

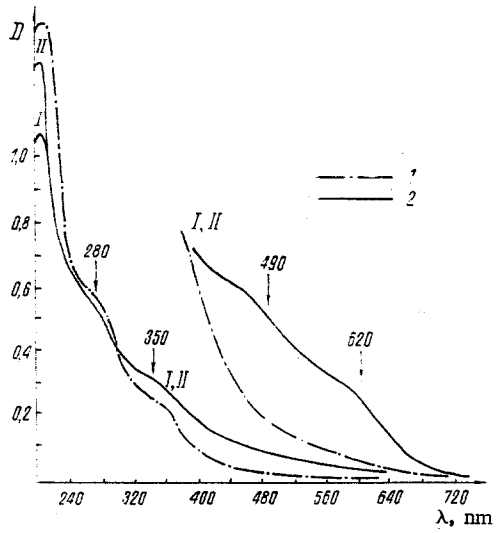

Fig. 5. UV spectra of the water-soluble melanins in water (after gel chromatography on Bio-Gel P-60): 1) brown; 2) black melanins. I) High-molecular-weight fraction; II) low-molecular-weight fraction.

This hypothesis enables us to interpret the changes in both the IR and the electronic spectra from a single point of view. In actual fact, the change in the protein–water intermolecular interaction to an interaction of a different type (protein–protein) enables the protein chains to form their inherent structure, i.e., to pass from a less ordered to a more ordered state. Apparently, the structural changes have two phases of transition: the first is transition from the brown melanin to the low-molecular-weight fraction of the black melanin. Here, judging from the parameters of the electronic spectra of alkaline and aqueous solutions, both the protein moiety of the pigment and the chromophore undergo rearrangements. This is shown in some decrease in the intensity of the $\pi - \pi^*$ band of the protein and increase in the intensity of absorption of the chromophore. The second stage is the passage from the low- to the high-molecular-weight fraction of the black melanin. Here the state of the chromophores scarcely changes, while the structure of the protein undergoes the far-reaching rearrangements described above. It is apparently not fortuitous that it is just in this stage that polysaccharide is found in the material.

EXPERIMENTAL

The UV spectra were taken on a Perkin-Elmer instrument and the IR spectra on a UR-20 instrument. The sample was molded into tablets with KBr (5 mg and 2.0 g, respectively).

Isolation of the Melanin. The melanin was extracted from the biomass of a culture of A. pullulans, strain 8, with 0.5 N NaOH for 1.5 at 0.5 atm with three changes of alkali to make the extraction more complete. The pigment was isolated and purified by known methods [9]. The work was performed with the true melanin fractions of the brown and black pigments.

Hydrolysis of the Pigments. The melanins were hydrolyzed with 6 N HCl in sealed tubes filled with nitrogen at 120°C for 24 h. After the end of hydrolysis, the unhydrolyzed part of the pigment was washed with water that had been distilled to a neutral pH, and with acetone and ether, and was dried. The amino acids in the hydrolyzate were determined quantitatively on a type Hd 1200 E amino-acid analyzer (Czechoslovakia). The amount of protein was calculated as the sum of all the amino acids. The brown pigment (5-day growth of the culture of A. pullulans, strain 8) and, particularly, the black pigment (18-day culture) were sparingly soluble in alkali after hydrolysis. Even in 3 N NaOH with heating they dissolved incompletely. Consequently, in the analysis of solutions of the pigments after hydrolysis we considered only the dissolved fraction.

Gel Chromatography. The brown and black melanins before and after hydrolysis were analyzed for homogeneity on a column (10 × 250 mm, free volume 5.5 ml) filled with Bio-Gel P-60. Aqueous solutions of the melanins were obtained by neutralizing their alkaline solutions with 6 N HCl to pH 7.0. On the column contain-

ing Bio-Gel P-60 were deposited 1-ml portions of a 0.1% neutral solution of the pigment before and after hydrolysis, and elution was performed with distilled water. The optical densities of all the fractions were determined on a SF-16 instrument at λ 475 nm.

The paper electrophoresis of the pigments before and after hydrolysis was performed in borate buffer (pH 9.25) at a potential difference of 300 V for 3 h. Three pieces of paper (type FN-11) were used for each sample. The first phoretogram was stained with fuchshin–sulfurous acid to reveal polysaccharide, the second with Bromophenol Blue to determine protein, and the third was left unstained as a control for the color of the melanin.

SUMMARY

1. On the basis of their IR and UV spectra and also a positive test with Ehrlich's reagent the brown and black pigments can be assigned to the melanins of the hydroxyindole type.

2. In the development process of the culture, the pigment undergoes a series of quantitative changes: the relative amount of protein decreases, the sample becomes nonhomogeneous, and colored products apparently of radical nature appear in the polymer.

3. As the culture ages, the structure and state of the melanin molecule change.

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