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STUDIES ON RHEOMELANINS. VI. THE APPARENT LIPOFUSCIN CHARACTERI--ETC(U)
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(6) STUDIES ON RHEOMELANINS,

VI. The apparent lipofuscin characteristics of rheomelanins *

by

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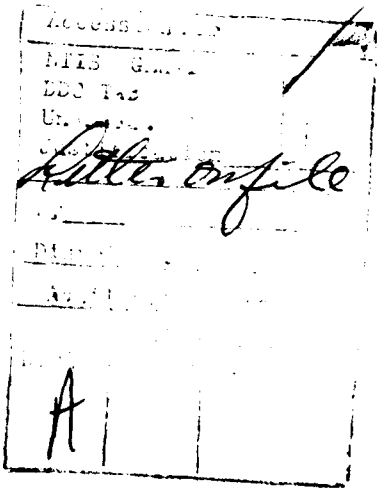
ABSTRACT

The positive histochemical tests obtained on rheomelanins indicate a relationship with tissue lipofuscins, which are also melanins in part. In addition chemical analysis indicates that the rheomelanins contain protein and lipid just as lipofuscins do. Fluorescence exhibited by the rheomelanins also seems to ally them to the lipofuscins.



INTRODUCTION

Earlier studies showed that rheomelanins (blood plasma soluble melanins) form during incubation at 37°C from the catecholamines, L-dopa and catechol in human whole blood containing added heparin, or potassium oxalate (Hegedus & Altschule, 1970a; Hegedus et al., 1971; Hegedus et al., 1978). Further work showed the apparent occurrence in vivo of rheomelanins in human blood (Hegedus, 1977). These rheomelanins (in vivo and in vitro) have characteristic yellow-green fluorescence when activated at wave-length of 366 nm, which seems to relate them to the solid lipofuscins rather than the solid melanins (Hegedus & Altschule, 1970a; Hegedus et al., 1971; Hegedus et al., 1978; Van Woert & Ambani, 1974). In order to study this further, slightly modified histochemical tests for solid lipofuscins in tissue slices and chemical tests for proteins, lipids and mucoproteins were applied. The results of this study are described below.

METHODS

Isolation of the Rheomelanins

The isolation was carried out in nitrogen atmosphere using deaired and nitrogen saturated solvents to avoid any oxidation. Eight 0.5 ml heparinized plasma samples were spotted on four Whatman No. 17 preparative chromatography paper strips. Each strip was approximately 54 cm long and 8 cm wide. These strips had been previously washed with distilled water and with the first developing solvent system (acetone:ethanol:H_2O - 1:1:18).

After spotting, partially drying the spots for 10 minutes and developing these chromatograms in a round glass jar under nitrogen, the yellow spot with the yellow-green fluorescence found at an Rf ≈ 0.75 in the almost dried paper was cut out and eluted with distilled water. The obtained impure rheomelanins after concentration to 4 ml were subjected to a second chromatography on another set of four Whatman No. 17 preparative chromatography paper strips, (same size as above), prewashed with water and the second developing solvent system (isopropanol: acetone:H_2O - 3:4:13). After spotting, partially drying the spots for 10 minutes, the chromatograms were developed under nitrogen. The yellow spot with the yellow-green fluorescence in the almost dried paper was cut out and eluted again with water. Approximately 45 mg of dried rheomelanins were obtained from 4 ml of plasma.

Chemical Composition Studies

In order to determine the relationship between the isolated plasma soluble fluorescent melanins and the lipofuscins, we have applied some of the histochemical tests used for solid lipofuscins in tissue slices to our isolated compounds. The isolated rheomelanins were subjected to the following tests:

1. Periodic Acid-Schiff Test
2. Long Ziehl-Neelsen Test for Acid Fast Lipofuscin
3. Alternative Nile Blue Method
4. Schmorl Test
5. Sudan Black B in Alcohol
6. Chrome Alum Hematoxylin Test
7. Protein Determination
8. Specific Mucoprotein Test
9. Phosphoric Acid-Vanillin Lipid Determination

The histochemical methods were slightly modified to be applicable to the isolated compounds on Whatman chromatography paper No. 1, silica gel on plastic sheets and on a porcelain plate. The procedure followed is described under each of the tests.

(1) Periodic Acid Schiff Test (PAS) - The rheomelanins in H_2O (ca1.0 mg) were spotted on a piece (1.5 cm square) of Whatman No. 1 chromatography paper and on silica gel on plastic sheet (0.2 mm thickness) without fluorescent indicator (E. Merck). They were dried and stored in the refrigerator for at least 24 hours for further drying.

The solutions for this test, namely the de Thomasi Schiff reagent, the periodic acid solution and the sulfite rinse, were prepared following the procedure for the test described by Culling in his book (Culling, 1963).

The well dried spotted samples were placed in distilled water for 30 seconds and then they were immersed in a 1% aqueous periodic acid solution for 3 min., washed in distilled water, immersed into the Schiff reagent for 20 min., rinsed 3 times with the sulfite rinse, washed with water, and left to dry. A positive reaction was indicated by the formation of a dark magenta color on both the paper and the silica gel plastic sheet.

No dark magenta color was observed using a blank chromatography paper, silica gel or control. Phenylether was used on the same kind of Whatman paper and silica gel plastic sheet as control to check for any non-specific adsorption of the color produced. Solid lipofuscins in tissue slices also give the magenta color by this method.

Spot tests were also carried out on a porcelain plate using the same reagents as above. Two processes were followed. In the first procedure, two drops of the isolated compounds (~0.5mg) were placed on the plate followed by two drops of the periodic acid reagent and left to oxidize for 20 min. This was followed by 4 drops of the Schiff reagent and 4 drops of the sulfite solution. The second procedure involved the addition of the sulfite solution before the Schiff reagent. A magenta color was obtained with both processes. A blank was used by substituting the isolated compounds solution with 2 drops of distilled water. Although the blank runs also showed positive results in the beginning, after the solutions were left to dry on the concave surfaces of the porcelain plate, different colors were obtained. The blanks showed a metallic yellow-bluish color while the samples showed a magenta color.

(2) Long Ziehl-Neelsen Method - This is another method utilized to identify tissue lipofuscins and to characterize them as "acid fast." A modification of the method described by Pearse in his book (Pearse, 1972a) has been followed. The sample, the control and the blank were prepared as were those for the previous PAS test, using Whatman paper and silica gel on plastic sheets. They were immersed in distilled water for 30 seconds and then placed in the carbol fuchsin solution for 3 hours at 60° C, washed with water and transferred to a 1% acid-alcohol solution. The silica gel plastic sheets with the sample showed a more intense magenta or red-violet color than the blank or the control. However, on the papers no differences in color among the sample, blank and control could be seen.

A spot test on a porcelain plate was also carried out. One drop of the solution of the isolated compounds was mixed with one drop of distilled water and one drop of carbol fuchsin solution. Phenylether and distilled water were used as control and blank, respectively. The porcelain plate was placed in an

incubator and kept there at 60° C for 3 hours. The sample developed a thick film of light magenta color. Phenylether gave a thin lilac colored film, while the blank developed a dark metallic blue spot. Solid tissue lipofuscin produces a bright red color when treated with carbol fuchsin solution. According to Pearse (Pearse, 1972b), any redness is considered a positive reaction.

(3) Alternative Nile Blue Method - A modification of the method described by Pearse in his book (Pearse, 1972c) has been followed. The samples, controls and blanks were prepared as were those for the previous PAS test. These were immersed in distilled water for 30 seconds and then they were soaked in the staining solution for 30 min. The staining solution was composed of 0.05% Nile Blue A in 1% sulfuric acid. The sample, control and blank were washed with water. The sample as well as the control and the blank were stained dark blue with equal intensity on both paper and silica gel. If washing the papers with water is omitted after staining, and they are washed instead with acetone or ethanol, the color is partially but equally removed from the blank, control and the sample. However on silica gel, washing with ethanol or acetone resulted in less removal of color, the sample's spot showing the greatest retention of blue color.

A spot test on a porcelain plate was carried out. Two drops of the solution of the rheomelanins were mixed with two drops of distilled water and then with four drops of the Nile Blue A staining solution. A blue solution was obtained. The blank and the phenylether control were also blue, however after one day standing at room temperature only the rheomelanin mixture remained blue upon drying, the blank turned brown and the control became colorless. Solid lipofuscin in tissue slices stains dark blue with Nile Blue A solution. Repetition of this spot test gave the same results.

(4) Schmorl Test - A modification of the procedure described by Pearse in his book (Pearse, 1972d) was followed. The ferricyanide and the ferric chloride solutions needed in this method were freshly prepared. The samples, controls, and blanks were prepared as were those for the previous PAS test.

The sample, control and blank papers were placed in distilled water for 1 minute and then they were immersed in the ferricyanide solution for 10 min, washed with water and dried. The sample's spot was stained blue, while the control and blank did not take the stain. Solid lipofuscins in tissue slices also stain blue.

Argentaffin granules, active sulfhydryl groups, melanin and lipofuscin are known to reduce the ferricyanide to ferrocyanide, which is blue. On the other hand, the sample spot on the silica gel sheet was stained bluish-green, while the phenylether control also stained dark blue. The blank did not show any color. The blue stain with the phenylether can not be explained.

Spot tests on a porcelain plate were also done. One drop of the solution of the isolated rheomelanins was mixed with 1 drop of distilled water and then with 1 drop of the ferricyanide solution. A green colored precepitate formed rapidly which a few hours later changed to dark blue, while the control and the blank developed a green color slowly which changed to blue overnight.

(5) Sudan Black B Test - This is a lipid staining test. The method described by Barka and Anderson in their book (Barka & Anderson, 1963) has been followed. The same kind of specimens as above were placed in 70% ethanol for 1 minute and then they were immersed in the Sudan Black B staining reagent for various periods of time: 1 min, 5 min and 20 min. They were then rinsed with 70% ethanol, followed by water. On the chromatography paper, the 1-min set comprised of sample, control and blank showed no difference in color. After 5 min and 20 min of staining, the sample developed a dark blue color. The control and blank were unchanged.

On silica gel plates, only the sample was stained blue after 5 min or 20 min of immersion in the staining solution.

Lipids and lipoproteins are known to stain black or blue in this method.

In the spot test one drop of 70% ethanol on a porcelain plate was mixed with one drop of the water solution of the isolated rheomelanins and then with one drop of the Sudan Black B reagent. This mixture gave a black color, which changed to dark blue in a couple of days. The control showed a purplish dark blue color which turned brown in a couple of days. The blank showed a black thin film which remained black upon drying.

(6) Chrome Alum Hematoxylin Test - The method for this test is described by Pearse in his book (Pearse, 1960). The same kind of sample, control and blank were used as those before. The papers and the silica gel plastic sheets were immersed in distilled water for 1 minute and they were then transferred into the acid permanganate solution for 2 min, and then bleached by immersion in 1% oxalic acid for 1 min. They were then washed with water and stained in chrome alum hematoxylin reagent for 10 min, and rinsed with water.

Only the samples were stained black-blue. The phenylether controls and the blanks were unchanged. Solid lipofuscin in tissue slices also stains black-blue with this method.

A spot test was done by adding to one drop of the sample one drop each of distilled water, acid potassium permanganate, 1% oxalic acid, and chrome alum hematoxylin, and mixing. The sample developed a purplish chocolate color which, upon drying, showed a black-blue color. The control showed a brown color and the blank showed a dark gray color upon drying.

(7) Protein Determination - Since lipofuscins are reported to contain protein, it was important to do a total protein determination on our isolated sample.

The general protein determination method of Lowry et al. was followed (Lowry et al., 1951). This method showed a relatively high protein content by the development of a dark blue color.

(8) Specific Mucoprotein Test - The procedure described by Grant & Kachmar (Grant & Kachmar, 1976a) using electrophoresis and staining has been followed. A concentrated solution of the isolated compounds was used (68 mg/ml).

The single yellow band obtained by electrophoresis was located around the middle of the cellulose polyacetatestrip. This band gave the purple color upon staining with Schiff's reagent, indicating the presence of mucoproteins in the isolated sample.

(9) Phosphoric Acid-Vanillin Lipid Determination - The procedure described by Ellefson and Caraway (Ellefson & Caraway 1976b) has been followed. A concentrated solution of our isolated compounds has been used (68 mg/ml). A pink color was obtained which indicates the presence of lipids in rheomelanins.

RESULTS AND DISCUSSION

We know little about the meaning of the different positive tests obtained with the rheomelanins. The positive periodic acid-Schiff test may mean the presence of carbohydrates and glycolipids, but it can also indicate the presence of aldehydes formed by the action of periodic acid on non carbohydrate containing unsaturated phosphatides. At present we know only that in the oxidation of lipids at certain intermediate stage Schiff-positive groups form (Pearse, 1972b).

The quality of acid-fastness was demonstrated using the long Ziehl-Neelsen method. This test might indicate the presence of unsaturated fatty acids (Pearse, 1972e).

The alternative Nile Blue method resulted in a dark blue color which is reported to be characteristic of lipofuscin (Pearse, 1972c). According to Lillie, the positive result is due to the presence of fatty acids (Lillie, 1936).

The Schmorl test proved to be positive with our isolated compounds which suggests the presence of groups that reduce ferricyanide to ferrocyanide (Pearse, 1972d).

The Sudan Black B test was positive, indicating the presence of lipids in our compounds. The Phosphoric acid - Vanillin reaction has also been performed for total lipid determination, and a characteristic pink color was obtained, although a relatively small amount of total lipid was found.

The Chrome Alum Hematoxylin test gave the black-blue color which is characteristic of lipofuscins.

The presence of relatively large amount of protein has been demonstrated by the method of Lowry et al. A specific test for mucoproteins using electrophoresis was also carried out and it was positive. Perhaps the relatively large amount of protein, the small amount of lipid in addition to the presence of mucoprotein in our compounds as compared to solid lipofuscin permits the rheomelanins to be soluble in plasma.

The rheomelanins are fluorescent and because of this it is possible to measure their concentration in blood plasma (Hegedus, 1977). Solid lipofuscins have fluorescence maxima in the 430-470 nm region and excitation maxima in the 350-380 nm region (Tappel & Fletcher, 1970). The excitation and fluorescence ranges and maxima of rheomelanins in distilled water are similar to these values (Hegedus, 1977). The positions of the fluorescence maxima of solid lipofuscins in solution are apparently uneffected by different solvents (Taubold et al., 1975).

It is not known so far what the role of lipofuscin is in human aging. What is known is that they accumulate with increasing age in the cells, in different amounts in the different organs, e.g. up to 75% of the cytoplasmic volume in large neurons, displacing vital organelles from their normal locations (Strehler, 1979). Since the rheomelanins appear to be lipofuscin-like substances, measurement of their concentration in blood plasma may provide a readily available scale indicative of the aging status in humans, independent of the chronological age. The lipofuscin described in all the studies on aging was solid, located in the cytoplasma of the cells of various organs and hence not accessible for quantitative measurements during life. The accumulation of lipofuscin, also called "age pigment", with increasing age is observed in protozoa, nematodes, drosophilia and other species.

Based on the dry weight of the pigment, it appears that solid lipofuscin is made up of 20-50% of lipid, 30-60% protein and 9-20% of hydrolysis resistant melanin (Miguel et al., 1977; Van Woert & Ambani, 1974; Van Woert et al., 1967). Research on solid lipofuscin suggests that unsaturated lipids in subcellular components are peroxidized by free radicals. Then lipofuscin is formed by condensation of the autoxidized unsaturated lipids with the products of progressive protein digestion, giving increasingly insoluble solid polymers (Miguel et al., 1977). Solid lipofuscins and rheomelanins exhibit electron spin resonance signals, indicating the presence of free radicals in both (Van Woert et al., 1967; Hegedus & Altschule, 1970b).

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