

XIV. THE EXTRACTION OF MELANIN FROM SKIN WITH DILUTE ALKALI.

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It has generally been assumed that the black pigments (melanins) which occur normally or pathologically in animal tissues, hair, skin, feathers, etc. are chemically very inert substances, and in most cases their isolation has been carried out by dissolving the tissue in boiling alkalis or strong acids. Thus Abel and Davis [1896] prepared melanins from both the skin and hair of a negro by heating the material at 100° either with 5-6 % potassium hydroxide or with concentrated hydrochloric acid until all the keratin had dissolved. Other investigators have used even more drastic methods such as boiling with more concentrated alkalis or even fuming hydrochloric acid.

That some melanins are really not so resistant to alkalis and acids was shown by Gortner [1910] who investigated the effects of alkalis upon the melanin of black-sheep's wool. He extracted the pigment from the wool by boiling with successive portions of a 0.2 % solution of sodium hydroxide, and from the first two or three extracts he obtained a melanin of constant composition, C = 52.57 %, H = 7.28 %, N = 13.43 %, S = 1.33 %. This substance when purified contained no ash, was soluble in alkali and in very dilute mineral acids ($N/20$), and was precipitated by stronger acids. After boiling this with stronger alkali a pigment could be precipitated by acid from the solution, which contained less nitrogen and hydrogen than the original, and was no longer soluble in dilute acids.

This acid-soluble pigment was only obtained from the first two or three extractions, the subsequent extracts yielding a different pigment, insoluble in acid and containing less nitrogen and hydrogen, and Gortner considered this to be a decomposition product of the acid-soluble pigment.

When stronger solutions of alkali were employed for the extraction, the compounds obtained were insoluble in acid, and their nitrogen and hydrogen decreased, in general, as the concentration of alkali increased; thus the substance extracted with 50 % sodium hydroxide contained only 3.84 % hydrogen and 8.98 % nitrogen.

So far from being inert to alkalis the melanin of sheep's wool was thus readily decomposed by boiling with sodium hydroxide in concentrations over 0.2 %, whilst even this concentration decomposed the pigment on continued boiling.

From these results Gortner considered that in all probability many of the melanins described in the literature were really products of the decomposition of melanin and not the pigment as it occurs in the natural material.

The melanin obtained by Abel and Davis from negro skin was left in the form of pigment "granules" (pigment and pigment structure) after the keratin had dissolved in the alkali; and the pigment was freed from the pigment structure by continued treatment with 5 % hydrochloric acid, and subsequent extraction with potassium hydroxide. It then had the composition C = 53.56 %, H = 5.11 %, N = 15.47 %, S = 2.53 %.

The author [Young, 1916] described the preparation of a melanin from the skin of an Australian aboriginal, by treatment with successive portions of boiling 5 % sodium hydroxide, each portion being allowed to act for a short time only. In this preparation no granules were obtained but the pigment was gradually dissolved in the alkali, from which it was obtained by precipitating with acid. The analysis differed from that of the preparation of Abel and Davis as it gave C = 60.12 %, H = 6.70 %, N = 11.89 %.

More recently an opportunity of obtaining more material came to hand, and further preparations were made, the method being modified so that a more dilute alkali ($N/20$) could be employed in the extraction. Pigments were extracted with this alkali from portions of the skin from two Australian aboriginals and a low caste Cingalee.

Preparation.

It was found that if the skin were soaked in boiling water, the outer layer, which contained practically all the pigment, could be readily removed by scraping. This layer (200 g.) was washed with alcohol and ether to remove grease, and was boiled with 100 cc. of $N/20$ sodium hydroxide for one hour under a reflux condenser. It was allowed to settle and the dark liquid decanted and filtered. The process was repeated with successive portions of fresh alkali until, after four extractions, there was only a very small residue. To the four clear filtrates after cooling, hydrochloric acid was added to about $N/3$, whereby the melanin was thrown down as a dark-brown precipitate. It was allowed to settle, the liquid siphoned off and the precipitate repeatedly washed by decantation with $N/3$ hydrochloric acid. The clear washings were siphoned off as much as possible and water and hydrochloric acid added to make the liquid to 500 cc. of $N/20$ hydrochloric acid. The mixture was then heated to boiling. The precipitates from the first two extracts completely dissolved to a deep brown solution; that from the third extract was only partially soluble; whilst the fourth precipitate did not appear to dissolve at all. The solutions were filtered through a fine filter paper (Schleicher and

Schüll's Blue Band), and the first three solutions mixed. In this way the pigment was separated into two fractions, one soluble, the other insoluble in dilute hydrochloric acid. The solution in hydrochloric acid was cooled, and concentrated acid added until the mixture was $N/3$. The melanin was thereby precipitated and was allowed to settle, the liquid siphoned off, and the residue washed several times by decantation with hydrochloric acid of the same strength. The process of dissolving in $N/20$ acid, filtering and reprecipitating was repeated once, the pigment was then again dissolved in the dilute hydrochloric acid, and the solution dialysed until the liquid no longer gave a precipitate with silver nitrate.

This treatment precipitated the pigment, which was then filtered off on a hardened paper, washed with distilled water, then with alcohol, finally with ether and dried. The paper was folded and extracted in a Soxhlet apparatus, successively with carbon disulphide, light petroleum and ether, and was dried at 100° . It was thus obtained as a black powder, and will be referred to as acid-soluble melanin. Before being dried the powder was readily soluble in dilute ($N/20$) sodium hydroxide, and in glacial acetic and concentrated sulphuric acids. It dissolved on warming in dilute ($N/20$) hydrochloric and acetic acids. After drying at 100° it dissolved only with difficulty in boiling dilute alkali, more readily in stronger alkali, and was almost insoluble in dilute hydrochloric acid.

The pigment from the later extractions of the skin, which was insoluble in dilute acid, was also purified by treatment on the filter paper repeatedly with boiling $N/20$ hydrochloric acid. It was then washed repeatedly with water and subsequently dissolved in warm $N/20$ sodium hydroxide. The melanin was precipitated by adding hydrochloric acid in slight excess, washed by decantation until free from acid, filtered and washed with alcohol and ether, and extracted as before with carbon disulphide, light petroleum and ether, and dried at 100° . This powder, after drying, dissolved completely, but only very slowly, in boiling alkalies.

In the above manner both acid-soluble and acid-insoluble preparations were obtained from the three skins.

Analysis of products.

The carbon and hydrogen were determined by burning in a current of oxygen by Dennstedt's method, lead peroxide being employed to keep back any oxides of nitrogen and sulphur. The yields of material were too small for a separate estimation of sulphur, and in some cases the sulphur was therefore estimated at the same time by Dennstedt's method. The quantity of barium sulphate actually weighed was very small, so that the results must only be regarded as approximate. Nitrogen was estimated by Kjeldahl, the heating with sulphuric acid being continued for four hours after the solution had become clear, as recommended by Dakin and Dudley with pyrrole compounds.

In one preparation nitrogen was estimated both by Kjeldahl's and by Dumas' methods with practically identical results.

In all cases before analysis the material was dried to constancy *in vacuo* at 100° over phosphorus pentoxide.

In some preparations which contained a high percentage of ash samples were again treated by suspending in *N*/3 hydrochloric acid in a dialyser, allowing the acid to dialyse away, and washing and drying as before, whereby the ash was very considerably reduced. In such cases the analysis before and after this treatment is given. The percentages are all calculated for the ash-free substances and the results are tabulated below.

	Sol. in dil. acid					Insol. in dil. acid				
	C	H	N	S	Ash	C	H	N	S	Ash
I. Aust. Black I:										
(a) ...	56.41	7.33	—	—	7.02	56.03	5.86	—	—	3.51
(b) After further treatment to remove inorg. matter	56.33	7.37	12.46	—	2.78	55.92	6.25	—	1.77	2.60
II. Aust. Black II:										
(a) Extracted by first portion of alkali ...	55.49	8.30	13.54	—	0.67	59.87	7.27	10.80	1.66	1.39
(b) Extracted subsequently	56.81	6.68	{ 11.95 12.24 }	2.49	4.03					
III. Cingalee:										
(a) ...	56.01	6.24	—	—	5.47	56.06	6.58	13.27	2.37	3.09
(b) After further treatment	56.06	6.71	14.57	2.56	2.68					
Previously found with 5 % alkali ...	—	—	—	—	—	60.10	6.70	11.89	—	—

In all cases the ash was left as a light brownish powder, only partially soluble in hot hydrochloric acid, and containing a small amount of silica. Estimations of the iron in the ash were made colorimetrically with potassium thiocyanate, and the quantity was found to vary from nothing in one sample, to 7 % of the total ash in another, the latter quantity corresponding to 0.2 % of the original pigment.

The iron was therefore not an essential part of the melanin molecule.

The ash also contained a trace of sulphate.

From the foregoing analyses it is seen that the ash was materially reduced by treatment of the melanin with acid, without altering the carbon and hydrogen percentage calculated on the ash-free substance. It would appear, therefore, that the ash is not an essential part of the melanin.

Action of alkali on the acid-soluble melanin.

A small quantity (about 0.5 g.) of the acid-soluble pigment from the Cingalee skin was boiled with *N*/20 alkali in a reflux apparatus. The sample which had been previously dried at 100° dissolved only slowly, passing, however, completely into solution after about five hours' boiling. The boiling was continued for 15 hours, after which the mixture was cooled, exactly

neutralised with *N* hydrochloric acid, and sufficient acid added to bring the whole to *N*/20. The melanin was partially precipitated, and even when heated to boiling some of the precipitate did not redissolve.

That this was not due to the drying alone was shown by warming another portion of the original dried melanin with the alkali until solution had taken place, neutralising and making to *N*/10 hydrochloric acid and boiling. In this case the melanin completely dissolved.

The pigment soluble in acid behaved, therefore, like Gortner's pigment, in that it was altered slowly by continued boiling with dilute alkali into a substance no longer soluble in dilute acid. Sufficient material was not available to test whether nitrogen and hydrogen were lost during this treatment.

DISCUSSION OF RESULTS.

It will be seen from the table of results that variations were found in the percentage composition of the different preparations. The acid-soluble pigment generally contained a lower carbon percentage and a greater percentage of nitrogen and hydrogen than the acid-insoluble pigment subsequently extracted from the same skin, although the insoluble preparations from one skin did not always contain less nitrogen and hydrogen than soluble preparations from another. It is observed also that in the case where the first extraction was kept separate the same difference was found between this and the pigment from subsequent extracts, although both pigments were soluble in acids. Continued boiling with dilute alkali converted the pigment soluble in acid to one insoluble in acid; it seems therefore probable that in extracting the pigment even with very dilute sodium hydroxide (*N*/20) it is gradually decomposed, losing nitrogen and hydrogen and gradually changing to a pigment insoluble in acid.

The melanin of black skin thus resembles that prepared from wool by Gortner in that a pigment may be extracted with alkali which is soluble in dilute acids, and which, on further heating with alkali, changes to a pigment insoluble in dilute acids, with loss of nitrogen and hydrogen. In this case, however, a constant product could not be obtained, and it is possible that none of the preparations represents the pigment as it occurs in the skin.

The preparations gave quite different analyses from the pigment from negro skin prepared by Abel and Davis.

REFERENCES.

- Abel and Davis (1896). *J. Exp. Med.* 1, 361.
Gortner (1910). *J. Biol. Chem.* 3, 341.
Young (1914). *Biochem. J.* 3, 460.