MELANOGENESIS, AND THE STRUCTURE OF THE MELANIN GRANULE.

Thesis presented for the degree of Master of Science in Physiological Chemistry at the University of the Witwatersrand, Johannesburg,

by

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November, 1954.
This is to certify that the work described in this thesis is original, it has not been presented for any degree at this or another University.

(Signed)   W.J. Stein

5th December 1954.
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MELANOGENESIS, AND THE STRUCTURE OF THE MELANIN GRANULE.

1. INTRODUCTION.

The black and brown pigments found in the animal kingdom are referred to as "melanins", the word being derived from the Greek "melas", meaning black. Some authors include certain red and yellow pigments in the class of the melanins. However, for the purpose of the present study, the term "melanin" will be considered as referring only to the black and brown pigments of animals.

There have been numerous investigations into various aspects of the problem of melanogenesis - the formation of melanin pigments by living organisms. In many tissues, including mammalian choroid, iris and certain tumours, the melanin pigment does not exist in an uncombined state but is contained in cytoplasmic particulates, the melanin granules. It was felt, therefore, that an analysis of the chemical composition of the melanin granule would lead to better understanding of the process of pigment production.

In this thesis, then, an account is given of an analysis of the chemical composition of melanin granules obtained from the choroid of the ox-eye. It will be shown that melanin granules do not consist merely of
pigment, but are structures having the complexity of other cell organelles such as mitochondria and microsomes. Evidence will be presented that the pigment of the granule shares a number of physico-chemical properties in common with the products of the oxidation of tyrosine by the enzyme tyrosinase. Information on the transformation undergone by tyrosine in its oxidative polymerisation to the synthetic melanin is reviewed in the light of the chemical studies on the melanin granule. An attempt will be made to assign roles in melanogenesis to the chemical constituents of the granule. On the basis of the results of the investigation, a mechanism for melanogenesis will be proposed. It will be shown that the available information on the structure of the melanin granule and on the properties of tyrosinase are equally consonant with two opposed views of the function of the granule. The formation of melanin pigment may be the primary function of the organelle, or alternatively the pigment may be an incidental by-product of a more important, although at present unknown, function of the granule.
SECTION II.

THE CHEMICAL COMPOSITION OF THE MELANIN GRANULE.

2.1 Isolation of the granule.

The choroid of the ox-eye, which tissue was chosen for the present investigation, contains in the cytoplasm of the melanin-containing cells, heavily pigmented granules, about 0.5 μ in diameter. On homogenisation by suitable methods, the cells of the choroid are disrupted and the melanin granules liberated. The granules can be freed from other cytoplasmic components, by differential centrifugation of the choroid homogenate.

Theory.

The technique of differential centrifugation was introduced by Bensley and Hoerr in 1934, and greatly elaborated by Claude, and by Schneider and Hogeboom.

In the last decade, differential centrifugation has become a widely used preparative procedure in biochemical investigations of the constituents of cells.
The acceleration of a particle in a gravitational field is given by \( A \) in:

\[
A = \frac{2}{9} \frac{r^2 p}{r_p} \sigma g
\]

where \( r_p \) is the radius of the particle, \( \sigma \) the difference in density between the particle and the suspension medium and \( g \) is the gravitational force applied.

Taking total differentials we obtain:

\[
dA = \frac{2}{9} \frac{r_p}{r_p} dr_p \frac{\partial A}{\partial r_p} + \frac{2}{9} \sigma \frac{\partial A}{\partial \sigma} + \frac{2}{9} g \frac{\partial A}{\partial g}
\]

\( dA \) is the increment in acceleration with changes in \( r_p, \sigma, g \) and is thus an index of the variation of the degree of separation with these variables.
The separation between two particles can be increased if the gravitational force is increased. For, from 2, \( dA \) is proportional to \( dg \). An increase in the density difference of two particles and the suspension medium will increase the degree of separation obtainable. Equation 2 indicates that differences in the radii of two particles will be more important for their separation than equivalent differences in the density of these particles. \( \sigma \) can have a positive or a negative sign according as the particulate has a greater or a lesser density than the suspension medium.

\( d\sigma \) will likewise have a positive or a negative sign and this will affect \( dA \). Use was made of this relation between \( d\sigma \) and \( dA \) in the present study - the purity of the melanin granule preparation was increased by manipulating the density of the suspension medium.

**Application to choroid tissue.**

Fractionation of pigment-containing tissue by differential centrifugation was first accomplished by Herrman and Boss (1945). Differential centrifugation has been applied to homogenates of melanomaiotic tissue. (du Buy et al. 1949). The work performed during the present study is the first attempt, to the author's knowledge, at a fractionation
of choroid tissue.

In preliminary experiments, the methods of Herrman and Boss (loc. cit.) and of du Buy et al. (loc. cit.) were applied to choroid tissue. Microscopical examination of the preparations obtained, after staining with methylene blue, revealed the presence of an appreciable contamination of the melanin granules with cell debris. The particles of cell debris were much larger than, but sedimented together with, the granule. Hence the cell debris was, presumably, of lower density than the granules.

(From Equation 2, \( d\sigma \) can be zero if \( \frac{2 + p\sigma}{\sigma} \frac{dA}{dp} = -d\sigma \frac{dA}{d\sigma} \) which is clearly possible for a particular \( d\sigma \) and \( dp \).

It was decided to take advantage of the high density of the granules, by sedimenting from a suspension medium intermediate in density between the granules and the cell debris. Only the granules would be sedimented, the cell debris being flung centripetally. (The idea of using this procedure arose in a discussion with my colleague, Mr. O. H. Callaghan, to whom I am greatly indebted). The validity of the procedure was confirmed by the purity of the granule preparation as established by microscopical observation.
Originally, the choroid tissue was homogenised in an homogeniser of the Potter-Elvejhem variety. It was found that the apparatus gave little disintegration of the relatively elastic choroid tissue. Later, a Waring Blender was used for the initial homogenisation. Two minutes in the Blender gave a shredding of the tissue but little destruction of the cells, - few melanin granules could be found in the supernatant fluid of a Blenderised preparation centrifuged at 600g for three minutes. Good yields of granules were obtained by grinding the Blenderised tissue with washed sand in a motor. Allowance was made, in the quantitative estimations on the granule, for contamination of the preparation by the dense sand particles.

The following procedure was finally adopted for the routine preparation of the melanin granules:-

One hundred choroids were carefully dissected out and placed into about four times their volume of ice-cold physiological saline. After disintegration by two half-minute runs in the Waring Blender, the homogenate thus obtained was ground with one-quarter of its volume of sand for two minutes. The whole was centrifuged for three minutes at 600g. The supernatant was kept aside, while the sediment was ground with sand for a further two minutes and the mixture respun. The process
of grinding and centrifuging was repeated five times. Further grinding
gave little increase in yield, although the final residue remained black-
grey in colour showing the presence of unremoved granules.

The combined supernatants were spun for three minutes at 600g,
and the sediment discarded. The granules were sedimented by spinning
the supernatant fluid for 30 minutes at 600g. This sediment was
re-suspended in 1.55 M sucrose (density = 1.2) and homogenised. From
this homogenate, the granules were sedimented by spinning for one hour
at 600 g. Although the supernatant solution, as evidenced by its colour,
contained many granules, it was pipetted off and discarded.

For the determinations involving the dry weight of the particulates
it was considered desirable to free the granules of occluded and adsorbed
sucrose. A sugar-free preparation was obtained by washing the granules
in distilled water, by repeated homogenisation and sedimentation. The
final sediment obtained was referred to as the "washed granule preparation" -
(WGP) - and used for subsequent analyses. The combined supernatant fluids
from the washing procedure were kept for analysis.

By the above method about 0.5 to 1 g of granules (dry weight) were
obtained from 100 choroids in 20 working hours.
2.2 Chemical Estimations on the Isolated Granules.

The washed granule preparation was analysed for the following components: pigment, lipid, ribose nucleic acid, non-diffusible protein, carbohydrate, total nitrogen and ash. In the ash, copper, zinc and iron were estimated.

The distilled water in which the melanin granules had been washed was analysed for dissolved protein and for ribose nucleic acid.

Many of the determinations were repeated on unfractionated choroid tissue, homogenised by treatment for two minutes in the Waring Blender.

The following Tables present the results of the chemical analyses:-
### Table I

Table I collects the results of the chemical analyses of the major constituents of the melanin granule.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Range of values obtained % (dry weight)</th>
<th>No. of determinations</th>
<th>Mean &amp; Std. deviation</th>
<th>Mean corrected for sand impurity</th>
<th>Diffused protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment</td>
<td>35 - 40</td>
<td>(4)</td>
<td>37 ± 2</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.9 - 5.0</td>
<td>(5)</td>
<td>2 ± 1.5</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>Non-Diffusible Protein</td>
<td>9 - 14</td>
<td>(4)</td>
<td>11 ± 2</td>
<td>13.5</td>
<td>11</td>
</tr>
<tr>
<td>Diffusible Protein</td>
<td>17 - 19</td>
<td>(3)</td>
<td>18 ± 1</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>8 - 13</td>
<td>(3)</td>
<td>11 ± 2</td>
<td>13.5</td>
<td>11</td>
</tr>
<tr>
<td>Ribose Nucleic Acid</td>
<td>△ 0.3 - 0.35</td>
<td>(3)</td>
<td>△ 0.33</td>
<td>△ 0.33</td>
<td>△ 0.33</td>
</tr>
<tr>
<td>Sand impurity</td>
<td>9 - 18</td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sand impurity from Nitrogen Analyses</td>
<td>7 - 15</td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ash (less sand impurity)</td>
<td>5 - 7</td>
<td>(3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fraction of the granule accounted for in the above analysis = 75 - 98%

Mean value = 86%

Therefore material undetermined = 14%
Table II collects the results of mineral analyses upon the ashed granules.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Range of values found in ( \mu g/g ) (dry weight)</th>
<th>No. of Determinations</th>
<th>Mean and standard deviation</th>
<th>Corrected for sand and diffusible Protein ( \mu g/g )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>27 - 54</td>
<td>(4)</td>
<td>38 ± 11</td>
<td>38</td>
</tr>
<tr>
<td>Zinc</td>
<td>560 - 870</td>
<td>(4)</td>
<td>680 ± 140</td>
<td>680</td>
</tr>
<tr>
<td>Iron</td>
<td>900 - 950</td>
<td>(4)</td>
<td>925 ± 25</td>
<td>925</td>
</tr>
<tr>
<td>Analyses on whole choroid tissue (dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>10 - 12%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigment</td>
<td>5 - 6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribose Nucleic Acid</td>
<td>1.3 - 1.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>6 - 7%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>7 - 9 μg/g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>200 - 300 μg/g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>140 - 160 μg/g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Microscopic examination of choroid tissue indicates that all of the pigment of the tissue is associated with the melanin granules. Choroid tissue contains 5-6% pigment. The melanin granule contains 37% pigment, therefore the proportion of melanin granules in choroid tissue is:

\[
\frac{5.5}{37} \times 100 = 15\%
\]

of the dry weight of the tissue. From the proportion of granules in choroid tissue and from the analytical data on the percentage composition of the granules, the amount of any constituent which is associated with the melanin granules in the choroid tissue can be calculated. Table IV presents the results of such calculations.
### TABLE IV.

**Distribution of Constituents between Choroid and Granule.**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Composition of granule.</th>
<th>Amount of constituent associated with granules in 1g choroid (dry weight).</th>
<th>% of constituent Associated with granule.</th>
<th>Enrichment factor.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>38 µg/g</td>
<td>6 µg</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>Iron</td>
<td>925 &quot;</td>
<td>135 &quot;</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>Zinc</td>
<td>680 &quot;</td>
<td>100 &quot;</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>Lipid</td>
<td>2%</td>
<td>0.3 g</td>
<td>3</td>
<td>1/5</td>
</tr>
<tr>
<td>RNA</td>
<td>0.3%</td>
<td>0.05 &quot;</td>
<td>3</td>
<td>1/5</td>
</tr>
<tr>
<td>Ash</td>
<td>6%</td>
<td>0.9 &quot;</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Pigment (assumed)</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>7</td>
</tr>
</tbody>
</table>

The fifth column gives the "enrichment factor", which is the ratio of the proportion of a particular component associated with the granule, to the proportion of granules present in the choroid. An enrichment factor of unity indicates that 15% of a constituent is associated with the granules, which comprise 15% of the material of the choroid, i.e. there is no differential concentration of the constituent between the choroid and granule. Higher values indicate that a constituent is concentrated in the granule, while lower values mean that the granule is deficient in a particular constituent.
2.3 Analytical Methods.

(a) PIGMENT.

Preliminary investigations disclosed that the major portion of the pigment of the melanin granule was soluble in alkali but was precipitated by acids from alkaline solutions. The alkali-soluble but acid-insoluble portion of the granule was defined as the melanin pigment fraction. The pigment fraction was therefore determined as follows:

The W.C.P. was rehomogenised in distilled water and made up to volume in a volumetric flask. Aliquots of this suspension were withdrawn and evaporated at 105°C to dryness, to obtain the dry weight of the granules present. To further aliquots was added sufficient 5 M potassium hydroxide to give a solution of final concentration 1 M. The potash solution of the granules was left at 39°C for 36 hours. The solution was centrifuged and yielded a dark-brown sediment and a dark-brown supernatant fluid. This sediment was re-extracted with M KOH at 90°C for 12 hours and again centrifuged. Extraction with potash was repeated until the supernatant solution was a pale brown colour. The sediment could thus be considered free from alkali-soluble pigment. On acidification of the combined potash solutions with hydrochloric acid the pigment precipitated as a red-brown mass,
leaving on centrifugation a red-brown supernatant solution not further precipitable by acid. This solution contained the "acid-soluble pigment." The acid-precipitated pigment was redissolved in M potassium hydroxide, the solution being subsequently centrifuged to remove alkali-insoluble matter. The supernatant solution was re-precipitated with acid. The process of solution in alkali and precipitation by acid was performed thrice. The third precipitate was washed three times with distilled water. The final sediment was dried at 105°C overnight and weighed. This weight gave that weight of the granule defined as "melanin pigment."

The property of solubility in alkali and complete insolubility in acid is unusual for biological substances. After three precipitations from alkaline solution, the pigment fraction of the granule attained a constant nitrogen value, which suggests that successive precipitates were identical. Washed, purified preparations of the melanin pigment yielded a nitrogen content close to the theoretical nitrogen content of "synthetic melanin" (See page 29). It is considered likely, therefore, that the pigment fraction contained all the melanin of the granule and little apart from melanin.
(b) **LIPID.**

Total lipid was estimated by extraction of the W.G.P. in a 3:1 chloroform:methanol mixture, as recommended by Hack (1953). The granules and solvent were left in contact for 24 hours at 25°C. The mixture was centrifuged and the supernatant solvent layer removed and kept. The sediment was re-extracted for 12 hours with chloroform-methanol in a Soxhlet apparatus. The solvents were united and evaporated at 80°C to 90°C to constant weight. The lipid-extracted granules were dried at 105°C to constant weight, and the weight of dry granules associated with the extracted lipid thus found.

On two occasions, Egeer's solution (3:1 ethanol:diethyl ether) was used in the above manner, with substantially the same results.

It is probable that the lipid extraction procedure removed most, if not all, of the lipid from the granules. Although about 15% of the granule was not characterised (Table I), nevertheless from the "nitrogen balance sheet" studies (Table V), it is evident that the non-characterised fraction contained about 25% nitrogen. The material not determined in the analysis of the granule is thus unlikely to be lipid. The extraction of 10 to 12% of the dried whole choroid tissue as lipid suggests that the solvents used were capable of dissolving large amounts of fatty material.
(c) **DIFFUSIBLE PROTEIN.**

An estimate of the total protein leached out of the granules during the process of washing with distilled water was defined as the percentage diffusible protein. The protein was determined as nitrogen by the Kjeldahl procedure. The washings gave no reaction with Bial's test for ribose nucleic acid.

(d) **NON-DIFFUSIBLE PROTEIN.**

Non-diffusible protein was estimated by determining the total amino-acid present in the supernatant solutions following extraction and precipitation of the melanin pigment. The amino-acid was determined by the "formol titration" method of van Slyke, described in Hawk, Oser and Summerson (1950).

Measurements of the amounts of the two protein fractions present in the granule were within limits, reproducible. It is considered, therefore, that this separation of the protein into two portions was a valid procedure.

(e) **RIBOSE NUCLEIC ACID.**

Ribose nucleic acid was isolated from the W.G.P. by the Schmidt-Tannhauser-Schneider procedure (Glick 1954). The ribose nucleic acid was estimated as pentose, using the orcinol reaction of Bial as
recommended by Umbreit (1949). Xylose was used as the pentose standard.

The ribose-nucleic-acid-containing fraction of the granule was pigmented, due to an acid-soluble pigment, which made difficult the determination of a lower limit to the ribose nucleic acid concentration. Thus, although it could be said that the ribose nucleic acid content of the granule was not more than 0.3%, it was not possible to determine the lower limit to this value with accuracy.

(f) **TOTAL CARBOHYDRATE.**

Determinations of the total carbohydrate present were performed on an alkaline hydrolysate of the granules, using anthrone in 76% sulphuric acid.

It is not possible to say how much of the carbohydrate found associated with the granules was, in fact, absorbed during the suspension in sucrose. The variation of the results obtained on different preparations was greater than the inherent variance of the estimation technique, suggesting that not all preparations contained the same amount of carbohydrate. Some, at least, of the granule-associated carbohydrate may have been introduced as an artefact during the preparation of the granules.
(g) **SAND IMPURITY.**

Two methods were developed for the estimation of the sand present as an impurity in the granule preparation.

a) The alkali-insoluble fraction remaining after the extraction of the melanin pigment (See p. 11) was well-washed with distilled water, sedimented by centrifugation, then dried at 105°C overnight, and weighed. The dried material so obtained was a light grey colour. The nitrogen content of this residue was determined. It was assumed that the nitrogenous matter was mainly pigment having a percentage nitrogen of 10%. Thus an estimate of the nitrogenous matter present could be obtained by multiplying the nitrogen content of the alkali-insoluble fraction by a factor of 10. Subtracting this estimate from the weight of the potash-insoluble residue gave the weight of sand present as an impurity in the granule.

b) A sample of granules was prepared in small yields by homogenising choroid tissue without the use of sand, in a Potter-Elvehjem type homogeniser. The nitrogen content of this preparation was determined. The percentage nitrogen
b) Contd:- yielded by a sample of the granules prepared using sand was found. The sand contained no nitrogen. Thus the difference in nitrogen content of the above two preparations could be used to determine the percentage sand present as an impurity:-

\[ 100 \times N_s = N_p (100 - x) + x \times 0 \]

where \( x \) is the percentage sand impurity

\( N_s \) the nitrogen content of the sand-prepared granules and \( N_p \) the nitrogen content of the granules free of sand and hence \( x \) could be obtained.

The two methods gave substantially concordant results.

(h) **ASH.**

Aliquots of the W.G.P. suspended in double-distilled water were washed in porcelain crucibles. Tenth-normal sulphuric acid was used to burn off the last traces of carbon. From the weight of ash found, the weight of sand impurity present had to be subtracted to find the true ash present. The sand was not volatilised in the ashing procedure.

The ash was estimated as the difference of two measurements, each subject to error. Thus the figure reported for the ash content of the granule should be considered as approximate only.
The minerals in the ash were extracted by boiling repeatedly with N/10 sulphuric acid for the copper and zinc, and with 50% v/v hydrochloric acid for the iron estimations. All solutions used in the mineral determinations were made up with double-distilled water, the second distillation being out of 'Pyrex' glass.

(i) COPPER AND ZINC.

The method of Bowness et al. (1950), with slight modifications, was used for the estimation of copper and zinc. This method determines copper and zinc as their diphenylthiocarbazone (dithizone) derivatives. Copper was extracted from the acid solution buffered at pH 4.75, also using dithizone in carbon tetrachloride. It was found that the concentration of the dithizone solution recommended by Bowness et al., namely, 0.003%, did not give a quantitative extraction of the copper ions. A solution of 0.015% dithizone was therefore used, with satisfactory results. Aliquots of the copper dithizonate solution were diluted with carbon tetrachloride before reading. The concentration of the solutions of copper and zinc dithizonate were read in a Leitz colorimeter, using Ilford filter No. 624 for the copper determinations and filter Nos. 625 and 607 for the zinc estimations.
The copper analyses showed a fairly wide variation, which was to be expected in view of the small samples used in the determinations. The technique did not allow of great accuracy with such small amounts of copper. A variance of ± 30% of the mean was found.

The zinc concentration of the tissue was much higher than that of the copper. But the greater accuracy in estimation which could therefore be obtained was offset by the inherently less sensitive nature of the zinc determination. A variance of ± 20% of the mean is reported.

(j) **IRON.**

Iron was determined by the method recommended by Hawk, Oser and Summerson (1950). The iron gave a red colour with potassium thiocyanate, extractable with amyl alcohol. The alcohol solutions were read in a Leitz colorimeter using Ilford filter No. 622. The technique used enabled the iron analyses to be made with an accuracy of ± 5% of the mean.

(k) **NITROGEN ANALYSES.**

The nitrogen content of the granules prepared with and without the use of sand to disintegrate the choroid tissue was determined. The nitrogen in the aqueous washings of the granules was estimated. The pigment fraction and the alkali-insoluble fraction of the granule, as well as the alkaline
solution containing the amino acids of the granule protein, were analysed for nitrogen. Thus it was possible to draw up a "balance sheet" for the distribution of the nitrogen of the granule over the various fractions into which the granule was divided (Table V). The nitrogen not accounted for by the lipid, pigment, protein and alkali-insoluble fractions of the melanin granule could therefore be established.

The mass of the granule not determined in the analyses reported in Table I could also be calculated. Thus the nitrogen associated with the undetermined portion of the granule could be estimated. It was felt that the nitrogen content of this not-accounted-for material would give an indication as to the nature of that material.
**TABLE V.**

"Nitrogen balance sheet"

<table>
<thead>
<tr>
<th>Nitrogen contents:</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanin granules, sand-free preparation</td>
<td>11.2</td>
</tr>
<tr>
<td>Melanin granules, prepared using sand</td>
<td>10.4</td>
</tr>
<tr>
<td>Melanin pigment</td>
<td>10.0</td>
</tr>
<tr>
<td>Undissolved (alkali-insoluble) residue</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Experiment of 8th June:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Nitrogen mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4 mg sand-free granules</td>
<td>3.8</td>
</tr>
<tr>
<td>gave 38 mg pigment</td>
<td></td>
</tr>
<tr>
<td>+ 12.5 mg non-diffusible protein</td>
<td>2.1</td>
</tr>
<tr>
<td>not accounted for</td>
<td>3.5 mg</td>
</tr>
<tr>
<td>i.e., 4.4 mg N/100 mg</td>
<td></td>
</tr>
</tbody>
</table>

Experiment of 6th August:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>mg N</th>
</tr>
</thead>
<tbody>
<tr>
<td>granules + sand</td>
<td>452</td>
</tr>
<tr>
<td>pigment</td>
<td>159</td>
</tr>
<tr>
<td>undissolved residue</td>
<td>75</td>
</tr>
<tr>
<td>non-diffusible protein</td>
<td>65</td>
</tr>
</tbody>
</table>

N not accounted for

i.e., 3.8 mg N/100 mg

17 mg
TABLE V

<table>
<thead>
<tr>
<th>Continuous:</th>
<th>mg</th>
<th>mg N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment of 27th August:</td>
<td>**</td>
<td>73</td>
</tr>
<tr>
<td>granules + sand</td>
<td>73</td>
<td>7.6</td>
</tr>
<tr>
<td>pigment</td>
<td>29</td>
<td>2.9</td>
</tr>
<tr>
<td>non-diffusible protein</td>
<td>7</td>
<td>1.2</td>
</tr>
<tr>
<td>alkali-insoluble residue</td>
<td>10</td>
<td>0.2</td>
</tr>
<tr>
<td>not accounted for</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

Total = 3.3 mg N

Undetermined nitrogen:

<table>
<thead>
<tr>
<th>Experiment of 8th June</th>
<th>4.4 mg N/100 mg granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot; 6th August</td>
<td>3.8</td>
</tr>
<tr>
<td>&quot; 27th August</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Mean = 4.1 mg N/100 mg granules.
(1) **THE ACID-SOLUBLE PIGMENT OF THE MELANIN GRANULE.**

It is evident from Table I, that for every 100 g of dry melanin granules, 15 g have not been accounted for as either pigment, lipid, protein, carbohydrate or ash. These 15 g are associated with 4 g of nitrogen (Table V) and have thus a nitrogen content of about 25%. This figure of 25% has been derived from the "balance sheet" studies, and is thus subject to a variation equal to the sum of the variations of the individual determinations involved in its estimation.

The 15 g of undetermined material included a dark-brown pigment which, unlike the melanin of the granule, was soluble in mineral acids. This acid-soluble pigment did not have the same ultra-violet absorption spectrum as melanin (See section 38). It was not found possible to estimate the amount of acid-soluble pigment present in the granule. This pigment may account for all, or only a small part, of the 15% of the granule which was not characterised.

The undetermined fraction of the granule possessed a nitrogen content which was in the region of that of proteins (15 - 18% N). However, this fraction did not react with formol and hence is unlikely to contain free amino acid. It may contain alkali-resistant peptides. The ultra-violet absorption spectrum of a solution of this uncharacterised material gave no indication of the presence of a high concentration of protein - there being no maximum in the U.V. curve in the region of 270 m\(\mu\). It may be that the peptides, if present, contain little tyrosine or tryptophan.
2.4 Comparison with the results of other workers.

Table VI compares the analytical data obtained by the author with the results reported in the literature.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Reference</th>
<th>Result</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper-whole choroid</td>
<td>Bowness et al. (1950)</td>
<td>9.8 µg/g</td>
<td>Present study</td>
</tr>
<tr>
<td>&quot;</td>
<td>Present study</td>
<td>8 µg/g</td>
<td>confirms earlier results.</td>
</tr>
<tr>
<td>Zinc-whole choroid</td>
<td>Bowness et al. (1950)</td>
<td>139 µg/g</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>Present study</td>
<td>150 µg/g</td>
<td></td>
</tr>
<tr>
<td>Ash-whole choroid</td>
<td>Bowness et al. (1950)</td>
<td>2.5%</td>
<td>Includes sodium chloride (1)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Present study</td>
<td>6.5%</td>
<td></td>
</tr>
<tr>
<td>Iron-whole choroid</td>
<td>Bowness et al. (1950)</td>
<td>Presence reported</td>
<td>No quantitative data (2)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Present study</td>
<td>200-300 µg/g</td>
<td></td>
</tr>
<tr>
<td>Ribose nucleic acid (granules)</td>
<td>du Buy et al. (1949)</td>
<td>&quot;high concentration&quot;</td>
<td>No quantitative data (3)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Present study</td>
<td>&gt; 0.3%</td>
<td></td>
</tr>
</tbody>
</table>
NOTES TO TABLE VI.

(1) The ash analyses on choroid tissue reported in the present study are higher than those reported by Bowness et al. (1950). The ash estimated in the present investigation included sodium chloride derived from the saline in which the tissue was homogenised. The presence of this salt may account for the higher ash analyses found here.

(2) Qualitative tests have indicated the presence of iron in choroid tissue (Bowness et al. 1950). The present investigation confirms that iron is present and provides a quantitative estimation of the amount of iron associated with the granule.

(3) There is a marked discrepancy between the ribose nucleic acid concentrations of the melanin granules obtained by du Buy et al. (1949), and those obtained by the author. This discrepancy may be explained by the fact that du Buy et al. did not extract ribose nucleic acid by the recommended Schneider-Tannhauser-Schmidt procedure, nor did they obtain quantitative data on the nucleic acid content of the granules isolated by them. It may well be, however, that the melanin granules of the melanomas studied by du Buy et al. have in fact a higher concentration of nucleic acid than have the granules
of the choroid. The nucleic acid content of tumour tissue is in general higher than that of normal tissue.

None of the remaining results obtained by the author has previously been reported.

2.5 **Summary.**

The dried melanin granule contains some 35% melanin pigment. 30% of the granule is protein of which two-thirds is soluble and the remainder insoluble in water. The lipid content of the granule is 2%, the ribose nucleic acid not more than one-third of 1%. Carbohydrate is present to the extent of 10% of the granule. Ash comprises approximately 5%. In the ash iron, zinc, and copper were determined. Iron formed 900 of each gram of dry granule, zinc 600/g. The copper content of the granule was .40/g.
SECTION III.

3. THE PIGMENT OF THE MELANIN GRANULE.

3. The Pigment of the Melanin Granule.

Some 35% of the melanin granule is composed of melanin pigment. The present study included an investigation into the nature of this pigment.

Many workers consider that the melanins of mammalian tissues are related to, or identical with, the black-to-brown compounds formed by the action of the enzyme tyrosinase on its substrate tyrosine (Fox 1953). A detailed comparison of these two groups of compounds, to the author's knowledge, has not been performed. The chemical properties of a natural melanin prepared from the ox-choroid; of a synthetic melanin formed by the oxidation of tyrosine by tyrosinase; and of a synthetic melanin obtained by the autoxidation of dihydroxyphenylalanine ("dopa"), a derivative of tyrosine, are compared in the following pages.

3.1 Preparation of the pigments.

a. Natural melanin.

The natural melanin was prepared from whole choroids, by a procedure similar to that used in isolating the pigment from melanin granules (page 11).
The pigmented tissue was refluxed with 2M hydrochloric acid for 50 hours. Much of the tissue dissolved to give a pale-coloured supernatant solution, the pigment remaining as an insoluble black mass. On boiling for 10 hours with 5M sodium hydroxide, most of the black residue dissolved to give a dark-brown solution. The alkali-insoluble residue was discarded. Acidification of the alkaline solution precipitated all of the dissolved pigment, a colourless supernatant fluid forming on centrifugation. Solution of the pigment in M sodium hydroxide and acidification with hydrochloric acid followed by centrifugal sedimentation of the precipitate was repeated until successive thrice-washed sediments gave constant nitrogen values on Kjeldahlisation. Three such precipitations were usually sufficient. The final precipitate was washed three times with distilled water and dried. The dried preparation is referred to as the isolated "natural pigment."

b. The synthetic pigments.

For the preparation of a "melanin" from tyrosine, the method of Raper and Wormald (1923) was followed.

The juice was expressed from minced potatoes and centrifuged to remove starch. 2g of tyrosine were dissolved in 4 litres of water. 400 ml of potato juice were added and the pH adjusted to 6.0 with a phosphate buffer. The solution was kept at about 37°C and stirred vigorously. After
five or six hours, by which time the solution had become dark red, 40 ml of 10% acetic acid were added and the solution rapidly boiled. The coagulated protein was filtered. The filtrate was made alkaline by the addition of 100 ml of 10% sodium carbonate and left to stand overnight. The resulting solution was black in colour. The pigment formed was precipitated by adding 200 ml of 10% acetic acid and boiling. The pigment was thrice dissolved in alkali and precipitated by acid as before, and then washed three times. This was the "acid-tyrosine-melanin" preparation.

An "alkaline tyrosine-melanin" was prepared by adjusting the pH to 7.8 after adding the enzyme. No red intermediate appeared under these conditions. The black pigment was precipitated, together with protein, on acidification with acetic acid, and purified by thrice precipitating and washing, as above.

A synthetic "melanin" was prepared from dopa according to the procedure recommended by Arnow (1938). Fifty ml of M/100 sodium hydroxide were saturated with dopa, and air was bubbled through the solution for two days. The solution quickly turned pink and subsequently blackened. The pigment was precipitated on acidification with hydrochloric acid and was thrice reprecipitated and washed, before drying. This will be referred to as the "Dopa-melanin" preparation.
3.2 Analyses of the Nitrogen contents of the three pigments.

A wide range of values for the percentage of nitrogen in natural and synthetic melanins has been reported in the literature.

Table VII compares the nitrogen contents of various natural and synthetic melanins prepared by the author and by others.
### TABLE VII.

**See Nitrogen of Natural and Synthetic Melanin.**

<table>
<thead>
<tr>
<th>Natural melanins</th>
<th>Method of preparation</th>
<th>( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author</td>
<td>Source of pigment</td>
<td></td>
</tr>
<tr>
<td>Serra (1946)</td>
<td>Black hair</td>
<td>7.0</td>
</tr>
<tr>
<td>Greenstein (1940)</td>
<td>Mouse melanoma</td>
<td>8.8</td>
</tr>
<tr>
<td>Waelsch (1932)</td>
<td>ox choroid</td>
<td>7.8</td>
</tr>
<tr>
<td>Present study</td>
<td>ox choroid</td>
<td>10.0</td>
</tr>
</tbody>
</table>

| Synthetic melanins                    |                                                               |         |
| Raper and Wormall (1923)              | tyrosine + tyrosinase                                           | 8.43    |
| Mason and Lada (1952)                 | Dopa + tyrosinase                                               | 8.18    |
| Bloch and Schaaf (1926)               | Dopa + tyrosinase                                               | 6.57    |
| Burton (1947)                         | Dopa + tyrosinase                                               | 7.8     |
| Present study                         | Tyrosine + tyrosinase                                           | 9.1     |
Elementary composition data are markedly affected by traces of impurity in the preparations analysed. Various workers have used different methods to prepare the natural and synthetic melamins listed in Table VII. It is not surprising, therefore, that a wide range of nitrogen contents has been reported for the pigments. On the basis of the nitrogen contents, it cannot be concluded that the natural and synthetic melamins are similar, but the table does not provide evidence to suggest the dissimilarity of the pigments.

Synthetic melamins, apparently, are slowly affected by atmospheric oxygen, yielding products of decreasing nitrogen content (Burton 1947). It has been suggested that peroxides are formed during the enzymic preparation of synthetic melamins and that these peroxides attack the melanin as soon as it is formed (Clemo et al. 1952, Swan and Wright 1954). Peroxidation of the melanin decreases the nitrogen content of the preparation finally obtained. The structure currently proposed for synthetic melanin (see page 52) should give a nitrogen content of 9.65%. The consistently lower values yielded by synthetic melamins may perhaps be explained as being due to peroxidation. The value of 9.65% is close to the 10% of nitrogen found in the natural melanin prepared during the present investigation by the relatively gentle method outlined on page 52.
No rigorous conclusion can be drawn from the similarity in nitrogen content of the natural melanin and the hypothetical synthetic melanin. However, the evidence does not cast out the possibility of the two pigments' being identical.

3.3 **Solubility and Colour.**

All three melanins dissolve in M/100 caustic alkali and, when concentrated, yield beautiful dark red-brown solutions. On dilution the colour fades to a pale yellow-brown. Acidification of the alkaline solutions precipitates the pigment in all three cases. The precipitate of tyrosine-melanin was more gelatinous than that of the other two melanins. The solubility in alkali and insolubility in acid of the melanins suggest their acidic (anionic) nature.

The colours of comparable concentrations of solutions of the three melanins appeared very similar to the naked eye. The colours of the solid pigments were apparently identical. The colour of a compound is considered to be due to the presence of a system of conjugated light-absorbing double bonds in the substance. Dark-coloured compounds such as the melanins possess structures capable of absorbing light at all wave lengths. The melanins thus contain numerous systems of chains of resonating double bonds. The similarity in colour of the three melanins suggests they contain closely
allied double bond systems.

3.4 Action of Oxidising and Reducing Agents.

The bleaching by hydrogen peroxide of melanin granules in tissue sections has been used as a test for the presence of the granules (Pearse 1947). It was found that isolated melanins were also bleached by hydrogen peroxide.

Comparable concentrations of natural-, tyrosine-, and dopa-melanins, dissolved in M sodium hydroxide, were treated with equal volumes of 30% hydrogen peroxide. Dopa-melanin was far more readily bleached than either natural or tyrosine melanin - a noticeable bleaching of dopa-melanin occurring after one hour, with complete decolourisation in three weeks. Natural melanin after three weeks of reaction was still yellow-brown in colour, the tyrosine melanin being only slightly paler. Bleaching was accelerated by boiling the reacting solutions. After four hours boiling, all three melanins had become completely bleached. The rate of bleaching was again: dopa-melanin > tyrosine-melanin > natural melanin.

The rate of bleaching is presumably a function both of the physical state of the pigment in solution, and of the chemical nature of the pigment. The slight differences observed during the peroxidation of the three melanins are thus not indicative of a significant difference in the structure of the pigments.
The use of Ehrlich’s test revealed that no indolaceous compounds were forming during the oxidative degradation of the melanins. It has been suggested that synthetic melanins are polymers of an indole derivative (page 52), hence it might have been expected that indolaceous compounds would result on the peroxidation of the pigments. Apparently, any indoles formed as intermediates during the degradation are further oxidised to simpler products.

On treatment of alkaline solutions of natural and tyrosine melanin with the reducing agent sodium hydrosulphite ("sodium dithionate") a vigorous reaction occurred with the evolution of garlic-like vapours. The melanins were bleached to buff-coloured compounds, bleaching occurring to about the same degree with both the melanins. No indolaceous compounds could be detected among the reaction products.

Conclusions:

Bleaching presumably consists of destroying the double-bond system responsible for the colour of the pigments. Hydrogen peroxide probably acts by oxidatively breaking open the double-bonds, while hydrosulphite may act by reducing these to single bonds. In both oxidation and reduction, therefore, the double-bond system of the melanins is destroyed. The similar behaviour of the melanins towards the action of oxidising and reducing agents suggests
that the pigments possess related double-bond systems - i.e. that the pigments possess related chemical structures.

DEGRADATION PROCEDURES.

It was felt desirable to attempt to disintegrate the molecules of the "melanin" pigments, using the classical degradative methods of organic chemistry. The preparations of natural and tyrosine melanins could not be assumed to be pure. Hence, for significant conclusions to be drawn, any breakdown products formed had to be obtained in large yields. Small yields of breakdown products could be argued to have arisen from non-melanin impurities in the preparation. However, if a degradation product of the presumably pure dopa-melanin was yielded, (even in small amounts) by natural melanin, strong evidence would be provided for the similarity in structure of the two pigments.

3.5 Zinc Dust Distillation.

Zinc dust distillation was used as a preliminary procedure in the present attempt at a degradation of the melanin molecule. Although the technique does not give unambiguous breakdown products, it often provides information which orientates further investigations. It was hoped that such an orientation would be provided here.
Method:

100 mg. of the finely powdered pigments were ground with about 500 mg. of fine zinc dust in a mortar. The mixture was placed in a hard-glass combustion tube, the remainder of the tube being packed with pure zinc dust. A stream of hydrogen, freed from hydrogen sulphide by bubbling through a concentrated potash solution, and then freed from potash by bubbling through water, was passed through the combustion tube. The opposite end of the tube was connected to a U-tube immersed in cold water. The hydrogen was ignited as it left the U-tube, in order to prevent an explosion. The combustion tube was strongly heated by four bunsen burners placed beneath the zinc and pigment mixture. Any volatile decomposition product would, it was hoped, collect in the U-tube.

Results:

Natural melanin on zinc dust distillation yielded vapours with a strongly phenolic odour, while a few droplets of a yellow fluid collected in the U-tube. These droplets were soluble in alcohol and formed a yellow fluorescent solution. The droplets were negative to Ehrlich's test for indole and derivatives.

Tyrosine melanin evolved a similar phenolic smelling vapour and one or two droplets of a yellow fluid collected as before. The droplets were again
soluble in alcohol giving a yellow fluorescent solution. This solution became pink on the addition of Ehrlich's reagent, indicating the presence of an indole unsubstituted in the β-position.

In the case of dopa melanin, again the phenolic odour was noticed, but no yellow droplets appeared during the zinc dust distillation. As an additional check for undetected yellow droplets the U-tube was washed with alcohol and the washings reserved for further analysis. These washings gave no colouration with Ehrlich's reagent.

Ultra-violet absorption spectra of the alcoholic solutions of the yellow droplets were obtained with the Beckman photometer. The spectral curves are depicted in Figure 1. As can be seen, the curves for the natural and tyrosine melanin breakdown products are very similar. The $\lambda_{max}$ are 254 $\mu m$ and 252.5 $\mu m$ for natural tyrosine melanin respectively, while $\lambda_{min}$ are 240 and 244 $\mu m$ in the same order. The washings of the dopa-melanin distillate were analysed in the ultra-violet. On one occasion the curve gave indications of a possible maximum in the region of 255 $\mu m$ but since this solution was apparently contaminated, the observation was not further considered.

The residue which remained in the combustion tube after zinc dust distillation, was treated with hydrochloric acid to remove the excess zinc.
FIG. 1. PRODUCTS OF ZINC DUST DISTILLATION.

A - NATURAL MELANIN

B - TYROSINE MELANIN
In all cases an alkali-insoluble tarry residue was obtained, apparently a result of charring during the reaction.

Discussion:

There were numerous differences in the behaviour of the three melanins towards zinc dust distillation. Thus, the natural and tyrosine melanins yielded yellow droplets having similar absorption spectra in alcohol, whereas dopa-melanin did not yield such droplets. Tyrosine-melanin evolved traces of an indolaceous compound; the other two melanins did not do so. Since the pure dopa-melanin gave no identifiable products on zinc dust distillation, it is probable that the products formed by the degradation of both natural and tyrosine melanin were due to impurities in the preparations. No conclusion can be drawn, therefore, from these zinc dust distillation studies.

3.6 Reduction with Hydriodic Acid.

As an alternative reductive method, the melanins were refluxed with concentrated hydriodic acid. No satisfactory method could be found to free the resulting reaction mixture from excess hydriodic acid, in order to obtain ultra-violet absorption curves. The melanins appeared to be destroyed during the reduction reaction. However, no indolaceous compounds could be detected in the reaction mixture.
A more promising attack on the problem of the identity of the natural and synthetic melamins had, in the meantime, been developed (see following section). Accordingly, the work with hydriodic acid was discontinued.

3.7 Potash Fusion.

Introduction:

It was noticed that on evaporating to dryness a potassium hydroxide solution of melanin granules, vapours of indole were evolved. This observation suggested that fusion of melanin pigment with potash might disrupt the pigment molecule to give indole or a derivative thereof. Potash fusion appeared to be a valuable degradative procedure.

Method:

Preliminary experiments showed that indole was liberated most strongly at a stage just before the boiling potash solution of melanin gave off the last traces of water. The following procedure was therefore adopted:

Natural melanin obtained by the repeated acidification of a potash solution of the isolated melanin granules was redissolved in a concentrated potassium hydroxide solution and placed in a flask fitted with a side-arm. About 100 mg. of pigment were dissolved in 5 ml. of the potash solution.
The flask and its contents were gently heated. The water distilled off and was condensed into a reservoir. Heating was continued until the contents of the flask had become quite dry. A further 5 ml. of water was added to the dry residue and the flask was reheated. This procedure was repeated three times. A steam generator was attached to the flask and steam was passed through the solution in the flask until about 50 ml. of water had distilled over into the reservoir. The collected distillate, which smelt strongly of indole, was kept for analysis. The residue in the flask was now almost completely colourless. The treatment with potash apparently degraded the melanin.

The distillate gave a strongly positive reaction with Ehrlich's reagent, a pink colouration being obtained indicating the presence of an indole nucleus unsubstituted in the position. An analysis of the amount of indole present in the distillate was performed using the method of Herter, as described by Hawk, Oser and Summerson (1948). In this method, sodium naphthoquinone monosulphonate in alkaline solution forms a coloured compound with indole, extractable with chloroform. Commercial indole was used as a standard.

The potash fusion was repeated using ovalbumen as a control.
Results:

**Natural melanin:**

Weight of pigment used = 58 mg.

Volume of distillate = 25 ml.

For standard solution of indole 0.8 mg in 10 ml, colorimeter reading = 75


distillate, 10 ml

" " = 14.5

Therefore weight indole in distillate = \( \frac{14.5}{75} \times 0.8 \times \frac{25}{10} \)

= 0.38 mgs.

Therefore percentage of indole = 0.65%.

**Ovalbumen:**

Weight of albumen = 200 mg.

Weight of tryptophan present = 2.14 mg.

Standard indole solution had 0.16 mg indole in 10 ml.

Colorimeter reading on Standard indole = 30

" " " total distillate = 10

Therefore indole in distillate = 0.05 mg.

Recovery of indole = 0.025% weight of protein

= 2% weight of tryptophan present.
Discussion:

At first sight, the amount of indole isolated as a breakdown product from the melanin seems insignificant. But, as can be seen from the experiment using albumen, the recovery of indole from melanin is of the same order as the recovery of indole from the tryptophan present in the protein. Potash fusions are known to give low yields of indolaceous breakdown products. Working with a tetrazolium compound, which contains an indole ring, Levy (1954) was unable to obtain more than 0.15% of the theoretical yield of indole, a figure again comparable with the yield of 0.65% obtained from melanin. It may be objected that the indole formed from the melanin preparation would, in fact, have arisen from tryptophan present as an impurity in the melanin. Again, the comparable experiment on albumen shows that the yield of indole is 25 times as great as would be expected had the entire melanin preparation consisted of a proteinaceous impurity having the same tryptophan content as albumen. Ovalbumen has a relatively high tryptophan content. There are few proteins which have a tryptophan content higher than 5%. Gramicidin is unusual in having a tryptophan content of the order of 20%. An estimate of the protein impurity in the natural melanin preparation can be obtained. If it is assumed that the pigment is a polymer of indole derivatives (see discussion on page 52), the pigment would contain 9.65% nitrogen, as compared with the 10% of nitrogen
actually found. This figure of 10% can be shown to be the nitrogen value of a mixture of 5% protein (having a nitrogen content of 16%) with 95% pigment. If the protein is further assumed to contain 5% tryptophan (a high value) then the tryptophan content of the melanin preparation is 0.25%. Assuming a quantitative conversion, 204 g of tryptophan would yield 117 g of indole. Thus a complete conversion to indole of all the tryptophan presumed to be present in the melanin preparation, would not account for more than one-quarter of the indole that was in fact evolved. And, it has been shown that the yield of indolaceous compounds during a potash fusion is closer to 1% than to 100%.

Thus it would appear that the indole evolved during the potash fusion of natural melanin arose from the degradation of the pigment itself and not from any impurity that might be present.

Efforts were made to increase the yield of indole by performing the potash fusion in a reducing atmosphere (zinc dust was added to the reaction mixture) and in an oxidising atmosphere (potassium permanganate being added). No higher yields of indole were obtained, the oxidising environment giving, in fact, a lower yield.
Conclusion:

The potash fusion degradation is very suggestive of the presence in natural melanin of an indole ring. This would seem to confirm the hypothesis that the natural melanins are related to the compounds derived from the oxidation of tyrosine. Higher yields of indole (in the vicinity of 5%) would place the hypothesis in less doubt. The decolourisation of the pigment during the potash fusion strongly suggests that the melanin has been degraded. If the degradation could be forced into paths leading to the formation of indole, higher yields of indole could be obtained. The formation of such higher yields has not yet been achieved.

3.8 Ultra-violet Spectra.

Introduction:

It was pointed out on page 30 that a black compound such as melanin is likely to possess a structure consisting of a system of conjugated double bonds, resulting in a generalised absorption of light at all wave-lengths of the visible spectrum. This generalised absorption does not preclude the possibility of differential absorption of light in the non-visible spectrum i.e. the ultra-violet (UV) and infra-red (IR). Also, it may be that slight inequalities of absorption at certain visible wavelengths might occur, not sufficient, however, to impart an obvious colour to the compound.
Subtler methods of observation than the naked eye might detect such inequalities.

For these reasons, it was decided to obtain spectrographic curves of the variation of light absorption with the wavelength of light absorbed, over the whole range of the light spectrum from the UV to the IR, insofar as the apparatus available allowed.

Method:

Solutions of the "melanin" pigments at comparable dilutions in M/100 sodium hydroxide, were read in the Beckman spectrophotometer, against M/100 sodium hydroxide as a standard. Curves were obtained in the UV from 235 m\(\mu\) to 400 m\(\mu\) and in the visible and near IR from 400 to 1000 m\(\mu\). The curves are depicted in figures 2 and 3.

Results:

The curves for the visible and IR spectra indicate a generalised absorption of light by the pigments at all wavelengths from 400 m\(\mu\) to 1,000 m\(\mu\). The spectrophotometric analysis does not yield any more positive information than is yielded by the naked eye observations on the colour of the pigments.

The UV curves, however, share in common a small bump in the
Fig. 2  ULTRAVIOLET ABSORPTION SPECTRA 

- - - - - - - - DOPA MELANIN IN $\frac{M}{100}$ NaOH
- - - - - - - - TYROSINE MELANIN IN $\frac{M}{100}$ NaOH, PH 6.8
- - - - - - - - NATURAL MELANIN IN $\frac{M}{100}$ NaOH.
- - - - - - - - TYROSINE MELANIN IN $\frac{M}{100}$ NaOH, PH 7.4
FIG. 3. VISIBLE & NEAR I-R ABSORPTION SPECTRA.

.. NATURAL MELANIN.  --- DOPA MELANIN.  --- TYROSINE MELANIN.
region of the curve between 270 and 290 m\(\mu\). In the case of dopa- and of natural melanin the bump seems to be maximum at 270 m\(\mu\), while for tyrosine melanin the bump is at 285 m\(\mu\). The slope of the dopa-melanin curve resembles that of natural melanin. The slope of the curve of tyrosine-melanin is not affected by the pH at which the melanin is formed. Tyrosine-melanin appears to differ slightly from dopa- and natural melanin.

Partial oxidation with hydrogen peroxide resulted in an increase in slope of the UV for tyrosine- and natural melanin. The bump was intensified and shifted to 260 m\(\mu\) (Fig 4). Acidification of the bleached solutions did not precipitate pigment and did not affect the absorption curves, beyond slightly diminishing the size of the bump. Extraction of the acidified solution with anaesthetic ether did not alter the form of the absorption curves. Dopa-melanin was not investigated. The partially oxidised pigment curves were more similar to one another than were the curves for the oxidised pigments.

Discussion:

Previous investigators have studied the UV absorption of various melanins. Curves published for UV absorption of dopa-melanin (Bowness et al. 1952, Clemo et al. 1952), and of tyrosine-melanin (Florence et al. 1935) depict bumps similar to those obtained in the present study. Florence et al.
FIG. 4. MELANINS BLEACHED BY HYDROGEN PEROXIDE.

- - TYROSINE MELANIN.  - - NATURAL MELANIN.
(loc.cit.) reproduce a curve for a natural melanin, which does not appear to possess a bump in the region of 270 to 290 μ, presumably due to a masking effect of the contaminants in a less pure preparation.

Of these three groups of investigators, only Bowness and his co-workers have commented on the bumps depicted in the absorption curves of their melanin preparations. Bowness et al. suggested that the bump was due to the presence of excess dopa and quote Mason (1948) in support of this suggestion. Mason demonstrated, however, that solutions of dopa do not give an absorption spectrum with a bump at 280 μ, after a short (three minute) exposure to air, although unoxidised samples of dopa do show a maximum in the absorption curve in the region of 280 μ. These observations of Mason were fully confirmed in the course of the present investigation.

While the melanins present a very similar picture on ultra-violet absorption analysis, it cannot be stated categorically that the structures of the pigments are similar. Not sufficient is known about those properties of a molecule responsible for light absorption to enable details of the fine structure of UV absorption curves to be interpreted unambiguously. At the present state of knowledge it can be affirmed, however, that the ultra-violet absorption analyses suggest that the melanin pigments studied possess related chemical structures.
SECTION III Conclusion:

The pigments behaved similarly to all the procedures applied. The Nitrogen contents, the solubility relations in acids and alkalis, the colour, and the behaviour towards oxidation and reduction of all three melanins were closely allied. The pigments demonstrated similar light absorption properties in the ultra-violet.

None of the above procedures is specific for the presence of a particular chemical grouping in the molecules studied. However, it is not likely that dissimilar compounds would resemble one another as closely as did the melanins.

The evidence presented above thus strongly suggests that natural melanin is related to the products of the oxidative polymerisation of tyrosine and its derivatives.
SECTION IV.

THE MECHANISM OF THE OXIDATIVE POLYMERISATION OF TYROSINE.

4.0 Introduction.

The evidence of the preceding section suggests that the natural melanins are related to the synthetic pigments formed by the oxidation of tyrosine and its derivatives.

Much information has been accumulated on the mechanism of the transformation of tyrosine into synthetic melanin. Such information is likely to be important for an understanding of the molecular transformations in melanogenesis. Only an outline of the available data will be given here, a full review having recently been published (Mason 1953).

4.1 Early Studies on Melanin Formation in Vitro.

The mechanism of the enzymic oxidation of tyrosine was investigated by Raper and his school in the 1920's (Raper 1928, 1932). Raper found that the following steps occurred during the oxidation:
DIAGRAM:

I \rightarrow \text{Tyrosine} \rightarrow \text{Dopa} \rightarrow \text{Dopa-quinone} \rightarrow \text{Melanins}

I: \begin{align*}
&\text{C}_6\text{H}_2\text{OH} \\
&\text{C}_2\text{H}_3\text{CH}_2\text{COOH} \\
&\text{NH}_2
\end{align*}

II: \begin{align*}
&\text{C}_6\text{H}_2\text{OH} \\
&\text{C}_2\text{H}_3\text{CH}_2\text{COOH} \\
&\text{NH}_2
\end{align*}

III: \begin{align*}
&\text{C}_6\text{H}_2\text{SO} \\
&\text{C}_2\text{H}_3\text{CH}_2\text{COOH} \\
&\text{NH}_2
\end{align*}

IV: \begin{align*}
&\text{C}_6\text{H}_2\text{SO} \\
&\text{C}_2\text{H}_3\text{CH}_2\text{COOH}
\end{align*}

2:3-dihydro-5:6 dihydroxyindole
-2-carboxylic acid quinone
It is generally accepted that the above steps are in fact followed during the preliminary stages of the formation of melanin. Thus the synthetic melanins appear to be polymers of derivatives of indole.

The reaction of tyrosine with tyrosinase proceeds very slowly in the absence of dopa - but with added dopa the reaction proceeds rapidly and smoothly (Mallette and Dawson 1929). The time-lag or Induction Period, as this phenomenon is called, is explained by the time required to form appreciable amounts of dopa by an initial slow reaction. As soon as sufficient dopa has been formed, the reaction proceeds rapidly. Lerner and Fitzpatrick (1950) put forward the following scheme to depict the action of tyrosinase on tyrosine and on dopa:

\[
\text{Tyrosine} \xrightarrow{\text{slow}} \text{Dopa} \xrightarrow{\text{fast}} \text{Intermediates} \xrightarrow{\text{fast}} \text{Melanin}
\]

Initially slow, but markedly accelerated, but small amounts of dopa.
4.2 The Polymerisation of the Indole Derivatives.

Raper and his colleagues did not study the problem of the manner of polymerisation of the indole derivatives that they isolated. This problem has been rigorously attacked in recent years and promises to yield valuable information as to the structure of the synthetic melanins.

Spectrographic studies by Mason (1948) suggested that the compound which finally polymerised to "Melanin" was the quinone of 2:3 dihydro - 5:6 dihydroxy indole (V) rather than the quinone of the related carboxylic acid (IV), as was suggested by Raper.

\[
\text{IV} \xrightarrow{3n^{++}} \text{V} \rightarrow \text{Melanin}
\]
The chemical studies of Beer et al. (1948) partly confirmed Mason’s view. Beer et al. showed that 5:6 dihydroxy indole was unstable in an alkaline medium giving rise to a dark brown product, whereas the derived -2-carboxylic acid was stable. Whether the presence of an enzyme would affect the stability of the carboxylic acid was considered, but not investigated, by Beer and his co-workers.

Mason and Wright (1949) showed that the transformation of IV into V was catalysed by zinc ions. This catalysis has been extended to yield a useful synthetic method (Harley-Mason and Bu’lock 1951). Decarboxylation of indole-carboxylic acids derived from IV can be catalysed by zinc ions. The catalytic activity of zinc is of particular interest, in view of the high concentration of the metal found in the melanin granule in the present study. The role of zinc in melanogenesis will be more fully discussed in Section V.

The mode of polymerisation of the indole-quinone V remained to be elucidated. Harley-Mason (loc. cit.) suggested that V, being a quinone as well as an indole, would be likely to possess the characteristic properties of both these classes of compounds. Indoles and quinones were known to condense together.

Harley-Mason and Bu’lock decided to investigate further the interaction of indoles and quinones as model systems of the possible manner
of self-condensation of the indole-quinone V. The conclusion was reached that 3-4 and 3-7 linkages between the indole nuclei forming the melanin were most probable.

\[
\text{\textbf{\textit{V}}}
\]

The possible structures having these 3-4 and 3-7 linkages were built in molecular models. It appeared that 3-4 linkages prevented the indole nuclei from attaining a coplanar configuration. This coplanarity was considered to be essential for resonance to occur, a condition suggested by the high light absorption of the melanins. Thus the following structure, having 3-7 linkages, was proposed:

\[
\text{\textbf{\textit{VI}}}
\]

Monomer has empirical formula
\[
C_8H_3O_2N
\]
\[\Rightarrow \% N = 9.65\]
The structure was considered to be cross-linked to similar chains at the 2-position (dotted arrows).

Harley-Mason and Bu'lock pointed out that a test of the validity of this structure would be provided by the investigation of the autoxidation of 5:6 dihydroxy-indoles substituted in the 7-position. Such a substituted derivative should not give a melanin, the formation of 3–7 linkages being prevented by the presence of the substituent.

Unfortunately for the hypothesis of Harley-Mason and Bu'lock, Beer et al. (1951) showed that both -7 and -4 substituted hydroxyindoles gave melanin-like precipitates on oxidation in alkaline solutions. Cromartie and Harley-Mason themselves synthesised a 7-hydroxyindole which also polymerised. Thus Harley-Mason's suggested mechanism for the polymerisation appeared to be untenable. All possible positions in the indole nucleus have since been substituted, but the results of studies of the derivatives on autoxidation, have been confusing and contradictory.

A "melanin" seems to be formed if the indole nucleus is substituted in any of the positions 1, 2, 4 or 7. Substitution in the 3 position yields products which turn blue to violet in alkaline solution but do not form "melanin."
It would seem then that the 3-position must be free for polymerisation to occur but either 3-\(\frac{1}{4}\) or 3-7 bonds can be formed to give a melanin polymer. This seems to contradict Bu'lock and Harley-Mason's coplanarity condition.

The manner of polymerisation of the indole-quinone V would be elucidated far more rapidly if a satisfactory definition of "melanin" was available; if sufficient was known of the chemistry of melanin to enable the pigment to be characterised. With the present state of knowledge, it is not possible to discover which of the polymerised indole-quinones are, in fact, true melanins. "It has now become clear that the rather crude methods used hitherto for the comparison of the (indole-quinone) autoxidations are inadequate." (Beer et al 1951). Not knowing which of the polymers are true melanins, it is not possible to determine from which substituted melanin the true melanins are derived. It is therefore impossible, at present, to ascertain the mode of polymerisation of the indole-quinone V and hence the structure of melanin cannot yet be formulated.
Section IV.

Conclusion:

The results of section 3.3 of this thesis, (the section dealing with the ultra-violet absorption spectra of the melanins) appear to have some bearing on the problem of the polymerisation of tyrosine to melanin. It will be recalled (Fig. 2) that, although all the melanins studied gave essentially similar curves, the curves for dopa- and natural-melanin were more closely related to one another than either curve was to that of tyrosine-melanin. Within the limits of the UV spectra method, these data suggest that the oxidative polymerisation of the precursor of natural melanin, in the environment of the ox choroid, proceeds in a manner more closely allied to the mode of autoxidation of dopa, than to the mechanism of polymerisation of tyrosine under the action of plant tryrosinase. However, the arguments put forward in the previous paragraph apply to the conclusions reached from a study of Fig. 2. Without possessing a method for characterising a melanin, it is not possible to distinguish between possibly different melanins. There is no method available for measuring the difference between tyrosinase melanin and the pigments derived from dopa or from the ox choroid.

The chemical investigations on the polymerisation of the substituted indoles open up an important field of study. Since various substituted
Indoles give "melanins", it may well be that natural melanin is formed from one of the substituted indoles rather than from the unsubstituted 5:6 dihydroxyindole. Methylation is a common biological phenomenon, and a mechanism may exist for the methylation of 5:6 dihydroxyindole prior to, or during the course of, the oxidative polymerisation of the compound. It would, therefore, be of value to study the methyl-substituted melanins, and their relation to the natural melanins.

It may be, too, that the polymerisation of differently substituted indoles yields natural pigments of differing properties. The indoles studied by the chemists have given mainly black, brown, violet and blue pigments an oxidative polymerisation. It is not too much to expect that further investigation will reveal yellow or red pigments formed by oxidative polymerisation of substituted indoles. The differences between the red, yellow, brown and black pigments found in nature might be due to differences in the degree of methylation (or substitution by other groupings) of the polymerising indole nuclei.
SECTION V.

MELANOGENESIS - AND THE STRUCTURE OF THE MELANIN GRANULE.

5.1 Introduction:

The melanin pigment of the ox choroid is closely related to the pigment obtained by the action of the enzyme tyrosinase on its substrate tryosine (Section 3). Furthermore, tyrosinase has been reported to be associated with the melanin granules of mammalian tissues (du Buy et al. 1949). It is therefore probable that the melanin of the choroid is formed by the tyrosinase associated with the melanin granules of that tissue. The role in melanogenesis of the components of the granule other than tyrosinase has not hitherto been considered. It will be the function of the remainder of the present discussion to consider the chemical components of the melanin granule in the light of the available information on melanogenesis. A brief review of this information will first be presented:

Melanin is formed by tyrosinase by the oxidative polymerisation of tyrosine. Thus a melanin-producing system must have access to tyrosine
and oxygen, as well as to the enzyme. Mammalian tyrosinase is considered to have no action on tyrosine combined in a protein (Lerner and Fitzpatrick 1950). Baker (1953) reports that free tyrosine is absent from the pigmented portion of frog skin. If this result of Baker can be considered to hold for mammalian tissues, then the formation of melanin by the mammalian melanocyte requires a system capable of liberating free tyrosine from protein. Presumably, cellular endopeptidases can be held responsible for this liberation of tyrosine.

Mammalian tyrosinase is a copper-containing enzyme (Lerner, Fitzpatrick, Calkins and Summerson 1950). Thus mammalian melanogenesis requires that copper be available to the pigment-producing system. Zinc has been shown to catalyse an intermediate reaction during the oxidative polymerisation of tyrosine in vitro (page 51). If tyrosine forms melanin in vivo by similar reaction sequences, it may be expected that zinc is required by mammalian pigment-forming systems.

The oxygen required for the oxidative polymerisation of tyrosine is, presumably, brought to the melanin-producing system of a cell by the cytochrome enzymes, which enzymes are involved in other cellular oxidations (Baldwin 1952).

In a stimulating contribution, Plummer and Kopac (1953) showed that the presence of tyrosine and tyrosinase only, in a system, was not sufficient for the production of melanin granules. "The transformation of pigment particles
into granules requires a physical substrate on which polymerisation and
deposition of the oxidised phenolic substances may take place."
"Even though the precursors of melanin might be present, along with the
enzyme, the polymerisation process must initially start on the surface of
a matrix, and so build up into a granule."

Thus it would appear, from the literature available, that the production
of melanin by a mammalian melanogenic system requires the presence of tyrosinase,
free tyrosine, oxygen, copper and possibly zinc, as well as the presence of a
structural matrix.
5.2 **Data.**

The following tables (VIII to XIII) collect some of the available data for the iron, zinc and copper and for the lipid and ribose nucleic acid contents of various tissues. The tables indicate which components of the melanin granule are abundant or scarce, as compared with other tissues or organelles. These indications suggest in which way the melanin granule is specialised. It may well be that such specialisation is associated with the melanogenic function of the granule.
**TABLE VIII**

Table VIII collates some values for the iron, zinc and copper content of various tissues and of some enzymes.

All values given in terms of dry weight of tissue.

<table>
<thead>
<tr>
<th>Iron</th>
<th>µg/g</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole man</td>
<td>60 - 100</td>
<td>Calculated from Harrow (1946).</td>
</tr>
<tr>
<td>muscle, beef</td>
<td>120</td>
<td>&quot;</td>
</tr>
<tr>
<td>heart, beef</td>
<td>250</td>
<td>&quot;</td>
</tr>
<tr>
<td>liver, beef</td>
<td>200 - 400</td>
<td>&quot;</td>
</tr>
<tr>
<td>foetal liver, beef</td>
<td>200 - 6,000</td>
<td>MacDougall (1947).</td>
</tr>
<tr>
<td>yeast</td>
<td>180</td>
<td>Calculated from Harrow (1946).</td>
</tr>
<tr>
<td>whole blood (dried)</td>
<td>85</td>
<td>&quot;</td>
</tr>
<tr>
<td>serum</td>
<td>8.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>3,300</td>
<td>&quot;</td>
</tr>
<tr>
<td>cytochrome</td>
<td>4,500</td>
<td>Paul (1951).</td>
</tr>
<tr>
<td>catalase</td>
<td>1,000</td>
<td>Therrell (1951).</td>
</tr>
<tr>
<td>cytochrome oxidase</td>
<td>800</td>
<td>&quot;</td>
</tr>
<tr>
<td>Iron</td>
<td>μg/g</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td>---------------</td>
</tr>
<tr>
<td>mitochondria, rat liver</td>
<td>200  - 400</td>
<td>Davidson 1950</td>
</tr>
<tr>
<td>microsomes, rat liver</td>
<td>200  - 400</td>
<td>&quot;</td>
</tr>
<tr>
<td>ox-choroid</td>
<td>200  - 300</td>
<td>present study</td>
</tr>
<tr>
<td>melanin granule</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>µg/g</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>whole man</td>
<td>4</td>
<td>Wintrobe e.a. (1953)</td>
</tr>
<tr>
<td>liver, human</td>
<td>35</td>
<td>Calculated from Wintrobe e.a. (1953)</td>
</tr>
<tr>
<td>liver, beef</td>
<td>150</td>
<td>Harrow (1946)</td>
</tr>
<tr>
<td>liver, human foetal</td>
<td>150 - 300</td>
<td>Wintrobe e.a. (1953)</td>
</tr>
<tr>
<td>muscle, human</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>nuts</td>
<td>11</td>
<td>Harrow (1946)</td>
</tr>
<tr>
<td>fish, whole</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>polyphenol oxidase, plant</td>
<td>3,000</td>
<td>Heilbrunn (1953)</td>
</tr>
<tr>
<td>tyrosinase, mammalian</td>
<td>2,000</td>
<td>Lerner et. al. (1949)</td>
</tr>
<tr>
<td>choroid, ox</td>
<td>8 - 10</td>
<td>present study</td>
</tr>
<tr>
<td>melanin granule</td>
<td>25 - 50</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE X.

<table>
<thead>
<tr>
<th></th>
<th>μg/g</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver, human</td>
<td>150 - 200</td>
<td>Neilbrunn (1953).</td>
</tr>
<tr>
<td>pancreas, beef</td>
<td>100 - 200</td>
<td>Harrow (1946).</td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>3,300</td>
<td>Neilbrunn (1953).</td>
</tr>
<tr>
<td>choroid, ox</td>
<td>150</td>
<td>present study.</td>
</tr>
<tr>
<td>melanin granules</td>
<td>550 - 700</td>
<td></td>
</tr>
</tbody>
</table>

Particularly striking are the values for Iron and Zinc. The concentration of iron the melanin granule is as high as that in the iron-containing enzymes catalase and cytochrome oxidase. Zinc, too, is present in high concentration, though sufficient data of a comparative nature are not available. The copper is high, but not remarkably so. It will be noticed that beef liver has three times the copper concentration of the melanin granule.
Tables XI and XII give the lipid and ribose nucleic acid contents of various tissues, on a dry weight basis.

<table>
<thead>
<tr>
<th>Ribose Nucleic Acid</th>
<th>µg/g</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>2,000 - 4,000</td>
<td>Calculated from Davidson (1950).</td>
</tr>
<tr>
<td>pancreas</td>
<td>4,000</td>
<td>&quot;</td>
</tr>
<tr>
<td>kidney</td>
<td>750</td>
<td>&quot;</td>
</tr>
<tr>
<td>brain</td>
<td>1,000</td>
<td>&quot;</td>
</tr>
<tr>
<td>muscle</td>
<td>150 - 300</td>
<td>&quot;</td>
</tr>
<tr>
<td>tumour</td>
<td>1,500</td>
<td>&quot;</td>
</tr>
<tr>
<td>mitochondria</td>
<td>800 - 1,600</td>
<td>&quot;</td>
</tr>
<tr>
<td>microsomes</td>
<td>6,800</td>
<td>&quot;</td>
</tr>
<tr>
<td>choroid, ox</td>
<td>1,500</td>
<td>present study.</td>
</tr>
<tr>
<td>melanin granules</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>% Lipid</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>newborn child (whole)</td>
<td>40</td>
<td>Calculated from Heilbrunn (1953)</td>
</tr>
<tr>
<td>sea urchin egg (whole)</td>
<td>21</td>
<td>&quot;</td>
</tr>
<tr>
<td>liver, beef</td>
<td>18</td>
<td>&quot;</td>
</tr>
<tr>
<td>kidney, beef</td>
<td>13</td>
<td>&quot;</td>
</tr>
<tr>
<td>pancreas, beef</td>
<td>35</td>
<td>&quot;</td>
</tr>
<tr>
<td>brain, whole, beef</td>
<td>25</td>
<td>&quot;</td>
</tr>
<tr>
<td>adrenal, guinea pig</td>
<td>50</td>
<td>&quot;</td>
</tr>
<tr>
<td>mitochondria</td>
<td>25</td>
<td>average of Table XIII, Section 5.3</td>
</tr>
<tr>
<td>microsomes</td>
<td>40</td>
<td>Claude (1949).</td>
</tr>
<tr>
<td>choroid, ox</td>
<td>10 - 12</td>
<td>present study</td>
</tr>
<tr>
<td>melanin granule</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

The melanin granule contains little RNA unlike that found in centres of active protein synthesis. The amount of RNA in choroid tissue, on the other hand, is higher than many tissues although lower than that found in tissues actively manufacturing protein.

The lipid content of the granule is low, that of the choroid also among the lowest recorded for animal tissues. The melanin granule contains the enzymes cytochrome oxidase and succinic dehydrogenase (Riley et al.1953), as also does the mitochondrion (Schneider and Hogeboom 1950). The melanin granules also possess tyrosinase (which is dopa oxidase) activity. Mitochondria appear to lack this activity.
5.3 Melanogenesis - and the structure of the melanin granule.

On the basis of the data of tables VIII - XII, it is possible to make some suggestions as to the possible function in melanogenesis of certain of the chemical constituents of the melanin granule. The components of the granule will be discussed in turn.

a) Proteins.

The melanin granule contains the enzymes tyrosinase, cytochrome oxidase and succinic dehydrogenase. It can be assumed that some of the protein of the granule is contributed by these enzymes. The remainder of the protein is likely to be performing as yet undiscovered enzymic functions, as well as performing a structural role.

The structural protein of the melanin granule is, presumably, in part contributed by the matrix which Plummer and Kopac considered to be essential for polymerisation of the melanin pigment.

It is possible that the mitochondria of melanin-producing cells may be the structural matrix upon which the melanin granule is built. This possibility will now be examined.

There has been lively discussion in recent years on the possible relation of the melanin granule to the mitochondrion, although the discussion has not been based on the concept of a "structural matrix" suggested above.
Du Buy and his co-workers hold that a relation exists between the melanin granule and the mitochondrion, and in fact term the melanin granules "melanized mitochondria" (du Buy et al. 1953). Other workers oppose this viewpoint (Dalton and Felix 1953). The chemical analyses on the melanin granule are pertinent to the discussion.

According to Woods (1953), "the mitochondrial nature of the melanised cytoplasmic granules is demonstrated by their highly specific activity for enzymes characteristic for mitochondria, (by) their oxygen-dependent staining with Janus Green B, and (by) the inverse numerical relationship between them and the number of amelanotic mitochondria in the same cells." In certain partially amelanotic melanomas, it is possible to find granules "in all stages of melanisation from a barely perceptible brown to a dark brown colour."

It cannot be assumed, however, that oxygen-dependent Janus Green B staining is specific for mitochondria. In fact, Lazarov and Cooperstein (1953) have shown that such Janus Green B staining is probably to be associated with the presence of the cytochrome oxidase system in the mitochondrion. Thus Wood's first and second points are equivalent:- The mitochondria and melanin granules contain a number of enzyme systems in common. Du Buy et al. (1953) report that the glycolytic activity of the melanin granules from the S91 melanoma was "in general comparable to the non-melanised mitochondria and sub-visible
particle fraction of hepatoma and liver. The differences noted were
quantitative. On the other hand, the melanin granules have some enzymic
activity not possessed by mitochondria. They have tyrosinase and hence dopa
oxidase activity (du Buy et al. 1949). It is not correct to assume that an
enzyme present in both the melanin granule and the mitochondria performs the
same function in each organelle. A possible function for the cytochrome
oxidase of the melanin granule, which function would not be required by the
mitochondrion, is suggested on page 74.

Dalton and Felix (1953) obtained electron micrographs of sections
of S91 melanoma tissue. They confirmed Woods' observation of the inverse
numerical relation between the melanin granules and mitochondria, but showed
that a similar relation held for melanized macrophages. These cells are not
considered to form their own melanin pigment; hence their mitochondria should
not produce melanin granules. Dalton and Felix were unable to confirm Woods'
finding that melanin granules exhibited different degrees of melanisation.
They further failed "to find a characteristic mitochondrial membrane associated
with melanin granules." Dalton (1953) reports that the mitochondria in the
cells studied were 0.2 μ in diameter and 1 to 7 μ in length, whereas the
melanin granules of the cell were spheres of diameter 0.2 μ to 0.4 μ.
Dalton's observations are evidence against a direct transformation of mitochondria into melanin granules. If a transformation did occur it would involve fragmentation of the mitochondria.

Smith (1920) in a study of the embryonic development of the chick choroid found no evidence of the direct production of melanin by mitochondria. In confirmation of this, Algard (1951) studying pigment cells in tissue culture under phase-contrast, could find "no evidence to support the hypothesis that mitochondria are converted into melanin granules."

Table XIII compares the composition of the melanin granules from the ox-choroid with the composition of rat liver mitochondria.
<table>
<thead>
<tr>
<th>Component</th>
<th>Melanin Granule</th>
<th>Mitochondrion and References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment</td>
<td>37%</td>
<td>-</td>
</tr>
<tr>
<td>Lipid</td>
<td>2%</td>
<td>26 - 29%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barnum and Huseby (1948).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27% Claude (1944)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29% Swanson and Aron (1950)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 - 24%</td>
</tr>
<tr>
<td>Ribose nucleic</td>
<td>0.3%</td>
<td>0.8- 1.6%</td>
</tr>
<tr>
<td>acid</td>
<td></td>
<td>Collected from Davidson (1950).</td>
</tr>
<tr>
<td>Protein</td>
<td>30%</td>
<td>63%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collected from Swanson and Aron (1950).</td>
</tr>
<tr>
<td>Iron</td>
<td>900 μg/g</td>
<td>200 - 400 μg/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collected from Davidson (1950).</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>11.2%</td>
<td>12.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collected from Claude (1943).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.5% &quot; &quot; &quot; (1944)</td>
</tr>
</tbody>
</table>
It can be seen that the chemical composition of the two organelles are significantly different. Especially noteworthy are the low concentrations of lipid and ribose-nucleic acid in the melanin granule and the high content of lipid and RNA in the mitochondrion. It is obvious that no direct transformation of mitochondria into melanin granules can be considered possible on the basis of the chemical composition data. It would seem incorrect to refer to the melanin granules as "melanised mitochondria". If, indeed, a mitochondrion does transform into a melanin granule, the profound change in chemical composition involved requires a drastic re-organisation of the contents of the melanin-producing cell. It appears desirable to regard the melanocyte as a whole, as forming the melanin granule, rather than to consider mitochondria as undergoing self-differentiation into the melanin granules of the cell. The mitochondria of the melanin-producing cells may form a part of the structural matrix that Plummer and Kopac (1953) consider to be necessary for granule formation. In the postulated process of transformation of the mitochondrion into the melanin granules, the mitochondrial nature of the matrix seems, however, to be lost.

b) Ribose Nucleic Acid.

The low RNA content of the granule may be correlated with the fact that melanin production does not involve the formation of peptide bonds. The melanin
granule may thus not be a centre of active protein synthesis.

Claude (1949) considers that high levels of RNA are always associated with high phospholipid concentrations. It may be that both RNA and phospholipid are involved in protein synthesis. In the melanin granule, the level of both RNA and total lipid is low and at least a part of the activity of the granule, namely the formation of melanin pigment, seems not to be concerned with protein synthesis.

The RNA content of the choroid tissue is higher than that of many tissues. Binkley (1954) considers that certain dipeptidases are polynucleotides or nucleoproteins. The relatively high nucleic acid content of the choroid tissue may be due, in part, to the presence of such nucleo-protein peptidases in the melanocytes of the tissue.

c) **Lipid.**

Calculations that the author has made show that there is sufficient lipid in microsomes, mitochondria and nuclei of cells to form a bimolecular layer of lipid over the entire surface of each organelle. In the microsomes, such a bimolecular layer accounts for 2/3 of their total lipid; for mitochondria, a bimolecular layer requires 2/5 of the lipid present in the organelle. 1/15 of the lipid molecules of the nucleus (including the lipid of the nucleolus)
would be necessary to form a bimolecular surface layer. It will be remembered that the lipid content of the erythrocyte was shown to be just sufficient to form a bimolecular layer over the surface of the cell. Furthermore, all the lipid of the red blood cell seems to be associated with the "ghosts" obtained after haemolysis. (Corter and Grendell 1925).

A similar calculation was performed for the melanin granule assuming it to be a sphere of diameter $0.3 \mu$ (the value found in electromagnetic studies) and containing 2% lipid. It was found that the lipid present was sufficient to cover only one-half of the surface of the granule with a bimolecular layer. Furthermore, it should not be assumed that the lipid present in biological structures forms a purely passive structural or storage role in the economy of the organism. Thus the surface-structural lipid of the melanin granule is not sufficient to form the double-layered lipid structure which has been assumed to explain the permeability properties of numerous biological systems (Davson and Danielli 1952). It may well be that the permeability of the melanin granule is not governed by the same relations as that of other cell organelles and of cells generally. In support of this contention is the work of Dalton and Felix (1953) which indicated that the melanin granule did not possess a membrane similar to that of the mitochondrion. Riley et al. (1953) consider the melanin granule to be unlike the mitochondrion
in that it is "remarkably resistant to visibly morphological change or damage when exposed to certain unphysiological conditions," such as changes in external tonicity. The melanin granule has not the fragility of the mitochondrion.

On the other hand, some protein was observed to be liberated when the melanin granules were transferred from hypertonic sucrose to distilled water (see section 2.3). Similar phenomena have been reported for mitochondria (Claude 1954). It would therefore seem that the melanin granule is sensitive to osmotic changes. A study of the osmotic relations of the melanin granule may well provide rewarding information as to the structure of the granule.

d) Minerals.

i. Copper.

Tyrosinase activity appears to be localised in the melanin granules of mammalian pigment-producing systems (see page ). Mammalian tyrosinase contains some 50 times as much copper as the isolated melanin granule (Lerner et al. 1951). Thus if 1/50th of the granule is tyrosinase, the total copper content of the granule would be accounted for. There has been no report of the tyrosinase content of the isolated granule but this figure of 1/50th is not unlikely. On the other hand, some tissues e.g. liver (Table IX) have a concentration of copper equal to, or greater than, that of the melanin granule - and possess no tyrosinase activity. Thus the copper in the melanin granule may be
performing a function other than that concerned with the tyrosinase activity of the granule. Copper has been found to be associated with the enzymes laccase, ascorbic acid oxidase and the so-called "green enzyme" concerned in the metabolism of acylated coenzyme A (Wintrobe et al. 1953). It does not seem likely that these activities can together account for the 150 μg/g copper in beef liver. It has been suggested that cytochrome oxidase contains copper (Eichel et al. 1950). This suggestion may explain the high copper content of liver. The melanin granule possesses cytochrome oxidase activity. It may be that some of the copper of the melanin granule is associated with this cytochrome oxidase. Quantitative estimations of the amount of tyrosinase and of cytochrome oxidase present in the melanin granule may throw light on the problem of the function of copper in the granule.

ii. Zinc.

Bowness and Morton (1953) investigated the influence of zinc and of other metals on the production of melanin by mammalian tyrosinase. They showed that during the formation of melanin in vitro, zinc was bound to a melanin-protein complex to the extent of 1.25%. They showed further that the addition of 100 μg of zinc doubled the light absorption of the melanin obtained from 1 mg of tyrosine. Zinc thus appeared to catalyse the production of melanin.
The significance of the zinc binding during the production of melanin is not clear. Iron also appeared to catalyse the formation of melanin, but only at an alkaline pH. 100 μg iron increased the light absorption of the melanin from 1 mg. tyrosine by 30%. Cobalt also appeared to perform a catalytic role in the production of melanin.

These catalytic effects are all very slight. A substance which doubles the rate of a reaction is not a particularly good catalyst. Furthermore, measurement of the light absorption of a melanin solution is not a good index of the activity of the preparation. The zinc may affect the light-absorbing or light-scattering properties of the solution investigated, without increasing the amount of melanin formed (Foster 1950).

Zinc catalyses the decarboxylation of the 5:6 dihydroxyindole - 2-carboxylic acid considered to be intermediate in the formation of synthetic melanin. This may be the explanation of the possible catalytic effect of zinc in Bowness and Morton's experiments and may also be the function of zinc in the melanin granule. Zinc has been found, however, to be associated with the enzyme urico-oxidase and with carbonic anhydrase. It would be of interest to determine whether the melanin granule possesses these or related enzymic activities. It does not seem likely that the function of the high concentration of zinc in the granule is the relatively slight catalytic activity reported by Bowness and Morton.
iii) **Iron.**

Iron is associated with many oxidising enzymes. None of these has been indicted in the production of melanin. The cytochrome oxidase system has been shown to be associated with the melanin granule. This enzyme is dependent upon iron for its activity (Heilbrunn 1953). An estimate of the amount of cytochrome oxidase in the melanin granule, using the results of Riley et al. (1953) and Slater (1949), shows that only 1% of the iron of the granule is associated with this particular iron-containing enzyme. It may be useful to investigate the presence in the granule of iron-requiring enzymes other than cytochrome oxidase. Clemo et al. (1953) reported that hydrogen peroxide is formed in a side-reaction during the formation of melanin by plant tyrosinase. If hydrogen peroxide is formed similarly during the production of melanin in the melanocyte, it may be that enzymes similar to the iron-containing catalase and peroxidase would be required to remove this hydrogen peroxide.
SECTION VI.

COMMENTS.

Less than 40% of the fully-formed melanin granule is composed of melanin pigment. This fact may necessitate a revision of the commonly-held view as to the function of the melanin pigments. It will be desirable first to discuss the function of the non-pigmented portion of the granule.

It has been shown (Section 5) that many of the components of the granule are such as to enable the granule to produce melanin pigment. Thus the granule contains tyrosinase, copper, zinc, a structural matrix, and cytochrome oxidase, all of which may play a role in the formation of the pigment. It is conceivable, therefore, that the fully-formed melanin granule actively produces melanin. The function of the melanin granule in the mature organism may be to store and to produce this pigment. It is equally conceivable, however, that the mature melanin granule performs a function other than primarily to produce pigment. It has been shown that tyrosinase may be concerned in the respiration of certain plants (Bonner 1950) and in the oxidative degradation of protein (Sizer 1953). Thus the function of the melanin granule in the mature organism may be other than to store and produce the pigment. These two views of the functioning of the melanin granule will be developed in the following pages.
If the function of the melanin granule is to store and produce pigment, the components of the granule can all be expected to perform some role in melanogenesis.

The chemical composition data on the isolated melanin granule lead, therefore, to the view that melanogenesis is a more complex process than has hitherto been supposed.

Previous views on melanogenesis (summarised on pages 57 - 59) do not explain either the high concentration of iron and zinc, or the low lipid and RNA content of the melanin granule, and do not account for the presence in the granule of enzymes other than tyrosinase. A more complex mechanism for melanogenesis will therefore be suggested, which mechanism takes into account these chemical composition data, as well as the information obtained by previous workers:

The melanin pigment is produced by the action of tyrosinase on tyrosine or a derivative thereof. Indole nuclei are formed as intermediates during the oxidative polymerisation of tyrosine under the action of the tyrosinase. These indole nuclei may be substituted in different ways by biological methyl-donating systems, giving rise to different types of melanins, and also, perhaps, to certain red and yellow pigments. Tyrosine is made available to the melanogenic system by the hydrolysis of tyrosine-containing proteins, this
hydrolysis being the function of cellular endopeptidases, possibly polymucleotides. Oxygen is brought to the melanin-forming systems by the cytchrome enzymes, perhaps by the cytchrome oxidase associated with the granule. Zinc catalyses an intermediate reaction during the oxidative polymerisation of tyrosine, but presumably performs other, at present unknown, functions. Hydrogen peroxide formed by a side-reaction during the formation of melanin is removed by the iron-containing enzymes associated with the granule. Iron may also catalyse directly the formation of melanin.

The pigment polymerises upon the structural matrix provided by the melanin granule itself. The finished melanin granule presumably possesses a weak protein-synthesising activity associated with the low RNA and phospholipid content of the organelle. The permeability of the granule does not appear to be governed by relations similar to those governing cells generally - the granule may be specialised to allow of the differential inward diffusion of tyrosine and of other substances involved in melanogenesis.

While it cannot be positively stated that the above mechanism in fact operates in melanogenesis, at least several alternative approaches to the problem are now open to investigation.

If it is considered that the melanin granule continuously produces
pigment, as a corollary it can be assumed that a continuous process of pigment destruction likewise occurs. Pigment metabolism in an organism would thus appear to be dynamic in character, the pigmentary state at any moment being a balance between pigment manufacture and pigment destruction.

If this view of the dynamic character of pigment metabolism is accepted, the mechanism proposed for melanogenesis should be extended to include the manner of pigment breakdown. The chemical behaviour of the melanins, as elucidated during the present investigation, indicates that pigment destruction can occur by oxidative or reductive mechanisms. It may well be that the minerals associated with the granules are concerned in pigment destruction, rather than in the formation of melanin. Thus if an oxidative mechanism is found to be involved in pigment breakdown, iron-containing enzymes may well be concerned in this oxidation, or in the removal of by-products of oxidation (e.g. hydrogen peroxide).

Models have been proposed to explain the action of such stimuli as the sex and adrenal hormones, and the effect of sunlight, on melanogenesis (Lerner & Fitzpatrick 1950). These models have been arrived at from a consideration of the pigmentary state of an organism as being due only to the activity of the tyrosinase of that organism. The concept of a dynamic state of pigment metabolism, in which both pigment production and pigment
destruction are considered to be complex, multi-enzyme processes, may well prove useful in the elucidation of problems in melanogenesis.

The modulating effect of hormones and of other factors on the pigmented state of an organism, may be mediated through systems other than the tyrosine-tyrosinase system. Hormones may depress the oxidative ability of the melanogenic system, or the tyrosine-liberating activity of melanin-producing cells, or yet again, may depress the catalytic activity of the zinc ions. The pigmentation of an animal may be increased, therefore, both by increasing melanin production and by decreasing destruction. The change in colour of an organism, which change is associated with certain clinical conditions e.g. pellagra, is also explicable by the concept of the dynamic state of pigment metabolism. It was pointed out on page 56 that differently coloured pigments may be formed by polymerisation of differently substituted indole nuclei. Thus an alteration in the rate of methylation of these indole nuclei may influence the colour of the pigmented organism. The rate of methylation is likely to be influenced by the hormonal balance, and hence by the clinical state, of the pigment-producing animal.

The chemical composition data of the present investigation are consonant, therefore, with a new concept of melanogenesis - with the view that pigment metabolism in organism has a dynamic and complex character.
It may be, however, that this concept of melanogenesis has been built upon an incorrect assumption; the assumption being that the function of the melanin granule in the mature organism is solely to store and to produce melanin pigment. This assumption presupposes that the melanin pigment of an organism is of value for the survival of that organism, that the pigment of the melanin granule itself possesses a physiological function. The pigment of dark-skinned races has been considered to be of value in the protection of the pigmented individual against harmful effects of the sun's rays. The pigment of the choroid and retina may have similar light-absorbing functions, connected with visual activity. It is difficult to conceive of a function for the black pigment of the substantia nigra, of Nissel's granules, and of the pigmented granules of amphibian liver. It is possible that in these latter environments, the melanin pigment is without function, being formed as an incidental by-product of the activity of tyrosinase, the most important function of the enzyme being other than to produce pigment.

The function of the melanin granule, therefore, may prove to be other than merely that of the continuous production of melanin pigment. The zinc, iron, copper and other constituents of the granule may be found to be concerned in this, at present unknown function of the granule. The association of melanin
pigment with the granule is conceivably a chance occurrence due to the
incidental property of tyrosinase that it forms black pigments from tyrosine.
By analogy, the presence of the enzyme urease in the Jack Bean, which plant is
never called upon to metabolise urea (Baldwin 1952), may be considered to be
such a chance occurrence.

The melanin granule may be presumed to possess at least two properties.
One property is the ability to produce melanin pigments; the other property
or properties confer upon the granule an unknown physiological function. In
these tissues of an organism where the melanin pigment performs a physiological
role, the production of pigment may be an essential feature of the metabolism
of the granule. In other pigmented tissues, production of pigment may be an
incidental by-product of the activity of the granule. In all pigmented tissues,
the granule may well be performing an important function, which function has
been overlooked because of the striking association of pigment with the granule.

Whether it will be shown that the production of pigment by the melanin
granule is the most important function of this organelle, or if it is shown
that the pigment is an incidental by-product of the activity of the granule,
it is certain that the melanin granule is an active, metabolising entity,
a complex, dynamic system. This concept of the active nature of pigmented
tissues may well be of value in providing a better understanding of the
physiology and pathology of melanogenesis.
SECTION VII.

SUMMARY.

a) Chemical composition data on the isolated melanin granule of the ox-choroid are presented.

b) It is shown that the melanin granule does not consist merely of pigment but possesses a structure having the complexity of the mitochondrion or the micorsome. Protein, lipid, nucleic acid, copper, iron and zinc are associated with pigment in the melanin granule.

c) It is shown that the pigment of the melanin granule possesses physico-chemical properties similar to those pigments formed by the enzymic and non-enzymic oxidation of tyrosine and of a derivative thereof.

d) A review is presented of the available information on the mechanism of the transformation of tyrosine into synthetic melanin. It is suggested that differences may exist between certain melanins, these differences being detectable spectrophotometrically, and being due, possibly, to a differential methylation of intermediate compounds during the polymerisation of tyrosine.

e) Possible roles in melanogenesis are assigned to the chemical constituents found in the melanin granule. An extended mechanism for melanogenesis is presented.
SECTION VII.

SUMMARY

Continued:

f)  It is demonstrated that the data on the structure of the melanin granule and on the properties of tyrosinase are consistent with two opposed views as to the importance of pigment production by the organism. The formation of melanin pigment may prove to be the most important function, or only an incidental by-product, of the activity of the melanin granule.
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