The Association of Zinc and other Metals with Melanin and a Melanin-Protein Complex

By J. M. BOWNESS and R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 18 *July* 1952)

The highest known concentrations of zinc in living matter occur in the melanin-pigmented tissues of eyes of some freshwater fishes (Leiner & Leiner, 1944; Bowness & Morton, 1952). Although the concentrations in pigmented eye tissues of mammals, amphibians, and saltwater fishes are a good deal lower, they are always higher than those in the unpigmented tissues of the same animals (Leiner & Leiner, 1942, 1944; Bowness & Morton, 1952; Bowness, Morton, Shakir & Stubbs, 1952). In the two last-mentioned papers it was shown that zinc and a number of other metals occurred in non-dialysable form in melanin-protein fractions from the eyes of cattle and perch. The accumulation in these fractions was sufficient to account for most of the zinc in the pigmented tissues.

The present work attempts to throw light on the combination between metallic ions and melanin or melanin-protein complexes. The interactions of zinc and other metals with various systems which transform tyrosine or 3:4-dihydroxyphenyl-L-alanine (dopa) to melanin have been studied.

Arnow (1938*a*) showed that a pigment could be obtained by bubbling atmospheric oxygen through an alkaline solution of dopa. Raper (1927) showed that dopa is an intermediate product in the oxidation of tyrosine to melanin by tyrosinase. It is probable that most animal melanins are synthesized by this series of reactions. As the absorption spectrum of the pigment produced by the oxidation of dopa resembles that of natural melanins (Arnow, 1938*b*) the structures are probably analogous, if not identical. The artificial pigment is therefore suitable for investigating the combination between metals and melanin in the absence of protein.

Winternitz (1918) showed that an extract of pig uveal tract catalysed the darkening of tyrosine suspensions, and Calkins (cited by Lerner & Fitzpatrick, 1950) reported the presence of tyrosinase and dopa-oxidase in ox ciliary bodies. It was therefore decided to try to extract from cattle irises and ciliary bodies an enzyme system catalysing the oxidation of tyrosine to melanin, and to use this system to study, *in vitro*, the effect of metals on melanin formed from tyrosine in the presence of protein.

MATERIALS AND METHODS

Reagents and apparatus. Reagents of A.R. grade were used whenever possible. All aqueous solutions were prepared with twice-distilled water, the final distillation being from, and into, Pyrex-glass vessels. All glass vessels were washed first with 50% (v/v) HNO_3, then with twice-distilled water, and dried in a stainless-steel oven. Subsequently they were scrubbed or rinsed in hot tap water, and then rinsed with twice-distilled water and dried.

Preparation of dopa-melanin solution. An aqueous solution of dopa (approx. 0·01%) was allowed to oxidize in air for several months. The resulting solution was diluted to approximately the original volume. On evaporating 10 ml. of the diluted solution at 50°, a dry residue (8·2 mg.) was obtained. The absorption spectrum of the solution is given in Fig. 1. The curve corresponds well with that given by Arnow (1938*b*) for the absorption in the visible region of his dopa-melanin. The small peak at 280 mμ. may be due to unchanged dopa (cf. Mason, 1948).

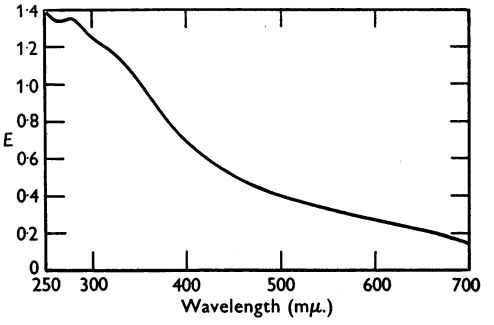

Fig. 1. Absorption spectrum of dopa-melanin solution in water (approx. 0·004% w/v).

Preparation of the extract from cattle irises and ciliary bodies. Fresh cattle eyes were obtained from the Liverpool abattoir and dissected within a few hours after death. A batch of twenty irises plus ciliary bodies was thoroughly ground in a mortar with crushed quartz, the pH of the mixture being adjusted to 7·2–7·5 by adding a solution of Na_2CO_3. The volume was made up to 200 ml. and the pH readjusted (7·2–7·5). The suspension was then usually allowed to stand for a day at 0° to permit maximum extraction of the enzyme. After standing, or sometimes immediately after dilution, the suspension was centrifuged until all the black material was precipitated. The supernatant was

filtered through glass wool and stored at 0° after readjusting the pH (7·2–7·5) if necessary.

Preparation of melanin from tyrosine. (Cf. Onslow, 1917.) In a 25 ml. Pyrex conical flask were placed the enzyme preparation (2 ml.), a 0·1 % suspension of tyrosine (1 ml.) and 0·05 % H_2O_2 (0·1 ml.). The flask was then lightly plugged with cotton wool and incubated at 38°. Blackening occurred even when H_2O_2 was omitted. Peroxide addition did not influence the absorption spectrum of the final product but reduced the induction period, i.e. the time between mixing the solutions, and the first appearance of blackening.

Fractionation of the extract from cattle irises. Various methods have been used to purify tyrosinase obtained from plant and animal tissues (Lerner & Fitzpatrick, 1950). In the present work a successful fractionation of the extract described above was achieved by only one of the standard methods, namely, fractional precipitation with $(NH_4)_2SO_4$. After each addition of $(NH_4)_2SO_4$, the pH was restored to 7 by means of Na_2CO_3 solution. Fractions precipitated by addition of 18–35 g. $(NH_4)_2SO_4$ (cryst.) to 100 ml. of extract were active, and by comparing the absorption curves of solutions obtained after 24 hr. incubation with tyrosine of various fractions, it was found that the material precipitated by 25–30 g. $(NH_4)_2SO_4$/100 ml. produced most melanin.

A standard procedure was used to obtain enzyme preparations for experiments to test the effect of metals on the formation of melanin. $(NH_4)_2SO_4$ (18 g./100 ml.) was added to the supernatant from ground irises and ciliary bodies, and the pH adjusted to 7 with Na_2CO_3 solution. The mixture was allowed to stand at 0° for 2–3 hr. and then centrifuged. The clear supernatant was separated and $(NH_4)_2SO_4$ (17 g./100 ml.) was added and the pH readjusted to 7. After standing for 3 hr. at 0°, the solution was centrifuged and the precipitate dissolved in twice-distilled water. The pH of the solution was brought to 7 by means of Na_2CO_3. The solution was then dialysed for 18 hr. at 0° against twice-distilled water containing Na_2CO_3.

Such dialysed solutions may be called enzyme preparations of type *A*. Preparations of type *B*, buffered to pH 6·8–7·0 with sodium citrate and NaOH, were obtained from type *A* by dialysing against a solution of sodium citrate and NaOH (pH 6·8) for 3 days at 0°, the outer solution being changed six times. This procedure removed all traces of carbonate from the enzyme preparation.

Estimations of zinc. The procedure described by Bowness *et al.* (1952) was used for all Zn analyses.

EXPERIMENTAL AND RESULTS

The interaction of dopa-melanin with metals

Preliminary experiments were carried out to test the effects of various metals on the rate of oxidation of dopa to melanin by H_2O_2. By measuring the absorption of red light by buffered solutions containing dopa, H_2O_2, and a metallic ion, at various times after mixing, it was found that Cu, Co, Fe^{2+} and Fe^{3+} accelerated melanin formation, the magnitude of the effect diminishing in that order. Zn, Ca and Mg had no effect, whilst Ba and Al had slightly inhibitory effects.

The effects of added metallic ions on the absorption curve of preformed dopa-melanin were then investigated.

Dopa-melanin solution (1 ml., see Methods section) was placed in each of a number of 20 ml. standard flasks; 3 ml. of water and 1 ml. of a solution containing 0·01 % of a metal (as sulphate or chloride) were added, and the flasks stoppered. A blank, containing 1 ml. of dopa-melanin and 4 ml. of water, was also prepared. After standing overnight the solutions were diluted to 20 ml. with twice-distilled water so as to give measurable intensities of absorption in the visible region.

The absorption curves between 400 and 700 mμ. for these solutions are shown in Fig. 2. In order to display the differences between the curves, the spectra in the region 250–400 mμ. have been

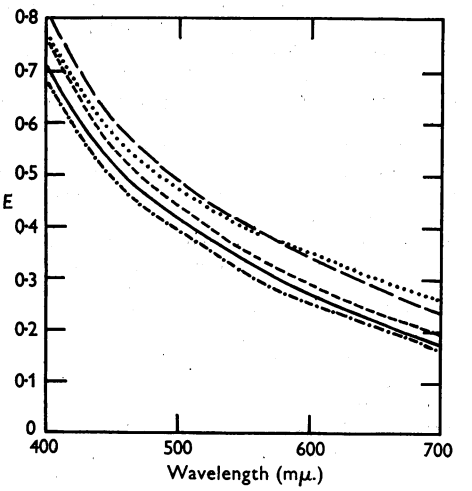

Fig. 2. Absorption spectra of dopa-melanin solutions (approx. 0·004 %, w/v) with added metals. (For details see text.) ·–·–·, dopa-melanin alone, dopa-melanin + $ZnSO_4$, dopa-melanin + $CaCl_2$, dopa-melanin + $MgCl_2$, dopa-melanin + $BaCl_2$; ——, dopa-melanin + $CoCl_2$; – – –, dopa-melanin + $FeCl_3$; — —, dopa-melanin + $CuSO_4$; . . . . ., dopa-melanin + $FeSO_4$.

omitted; it is notable, however, that the small peak at 280 mμ., shown in Fig. 1 for the untreated dopa-melanin, also occurred in the solutions to which metals had been added, except in the case of copper, where it appeared as an inflexion only.

The amount of zinc combining with protein from a cattle iris, and with a complex between this protein and melanin

The aim was to find out how much of a given excess of zinc was taken into non-ionic combination with (*a*) melanin formed in the absence of protein, (*b*) melanin formed in the presence of protein, and (*c*) the same protein without melanin.

An enzyme preparation from cattle irises and ciliary bodies was used for the formation of melanin from tyrosine and the protein was obtained by adding 10 g. $(NH_4)_2SO_4$ to 100 ml. of the supernatant of the suspension obtained in the manner described earlier. The pH was adjusted to 7 with

Na_2CO_3, and the precipitate centrifuged down. A further 20 g. of $(NH_4)_2SO_4$ were added to the supernatant, and the pH again adjusted to 7. The resulting precipitate was centrifuged down and a 5 % (w/v) solution of this material in phosphate buffer, pH 6·8, was made up. The reactants were placed in three 250 ml. beakers; the ratio of surface area to total volume of reactants was then large enough to permit ready absorption of O_2. Beaker *A* contained 10 ml. tyrosine suspension (0·1 %), 10 ml. citrate buffer, pH 6·8, containing 2 mg. Zn^{2+} as $ZnSO_4$ and 2·5 mg. of CO_3^{2-} as Na_2CO_3, and 5 ml. of 0·1 % dopa solution. Beaker *B* contained 10 ml. tyrosine suspension (0·1 %), 10 ml. citrate buffer, pH 6·8, containing the same amounts of $ZnSO_4$ and Na_2CO_3 as in beaker *A*, and 5 ml. of the protein solution described above. Beaker *C* contained 10 ml. of twice-distilled water, 10 ml. of citrate buffer (as for *A* and *B*) and 5 ml. of the protein suspension used in *B*. The three beakers were incubated at 38° for 4 days, diluting each with 10 ml. of water on the second day. On the fourth day 50 mg. trypsin (British Drug Houses) were added to beakers *B* and *C*, and incubation continued for a further 3 days. Beaker *A* was left in the incubator at 38° for a further 30 days (diluting with twice-distilled water at intervals), when the formation of pigment appeared to have ceased.

After trypsin digestion, the contents of beakers *B* and *C* were centrifuged, first at low speed to bring down the fraction which separates with the pigment, then at high speed to bring down as much protein as possible. The pigment fractions were washed and re-centrifuged several times, and then dried, weighed, ashed, and analysed for Zn. The rest of the protein was discarded. The supernatants were evaporated at 100° to about 10 ml. volume and centrifuged again; the precipitates were discarded. Acetate-acetic acid buffer, pH 4·75 (10 ml.), was added to each supernatant, and the volume of each mixture made up to 50 ml. with twice-distilled water. Two 0·5 ml. samples from each solution were taken for Zn analysis. Each sample was diluted to about 7·5 ml. with twice-distilled water and shaken with excess CCl_4 in several portions. This precipitated the remaining protein and removed it by adsorption at the interface. The CCl_4 layers (droplets) were run off, and the Zn extracted from the solution by dithizone in CCl_4, after adding 2·5 ml. more acetate buffer, and 1 ml. of $Na_2S_2O_3$ solution. After 30 days' incubation, beaker *A* was treated in a similar way, except that no trypsin digestion was used. Hardly any material precipitated during centrifugation. The results are given in Tables 1 and 2.

It is evident from this experiment that no zinc is bound in non-ionic form by melanin formed in the absence of protein, some is bound by the protein fraction from cattle irises and ciliary bodies, but much more is bound by a complex of this protein with melanin formed from tyrosine.

It is probable that the 10 mg. of tyrosine present in beaker *B* were not entirely converted to melanin. Assuming, however, that the conversion was complete, the minimum concentration of zinc in a pigment-protein complex analogous to those isolated from cattle and perch eyes can be calculated. The melanin in these complexes was combined with roughly its own weight of protein (Bowness *et al.* 1952); the total weight of pigment-protein complex must therefore be a maximum of 20 mg. The amount of zinc combined by this material must be 250 μg., giving a concentration of 1·25 %. This is of the same order as the zinc concentrations found in the pigment fractions from frog and perch eyes (2·8 and 4·38 %, respectively).

Table 1. *Amount of ionic zinc in solutions of melanin formed from tyrosine and dopa, protein from cattle irises, and mixtures of the two*

Beaker	Total ionic Zn in solution (μg.)	Total bound Zn (μg.)
A	2000	—
B	1550	450
C	1800	200

Table 2. *Zinc concentrations in the pigment fractions separated by slow centrifugation of the products of incubating tyrosine with protein, and protein alone*

Beaker	Dry wt. (mg.)	Zn concentration (μg./mg.)
A	—	—
B	18·7	4·6
C	9·7	0·83

Oxidase activity of the enzyme preparations from cattle eyes

In preliminary experiments it was shown that enzyme preparations of types *A* and *B* (see Methods) catalysed the uptake of oxygen by tyrosine and dopa solutions (as measured by a Warburg technique) while melanin formation was in progress.

Influence of various metals on the absorption curves of pigments formed from tyrosine in the presence of the enzyme system from cattle irises and ciliary bodies

The reaction mixture and conditions necessary to produce melanin from tyrosine have been described already. Various metals were added at the start of the reaction, and the absorption spectra of the solutions compared when melanin formation had reached its limit. Measurement of gross light absorption by the solutions (using a photoelectric colorimeter) showed that colour production had usually reached completion after 1–2 days, and always within 3 days. If the solutions were left for 6 or 7 days after mixing, the pigment particles began to agglomerate, and the solution lost its colloidal character. For 4 or 5 days the particles of pigment were too small to be readily precipitated by high-speed centrifugation. After 5 or 6 days the effects of contamination by various organisms often become apparent. It was decided therefore to take absorption readings 3 days after mixing the solutions.

Another set of flasks was prepared as described earlier. The volume of liquid in each flask was made

up to 4·1 ml. by the addition of 1 ml., or less, of a 0·01 % solution of a metal (as chloride or sulphate) and a corresponding volume of water. After 3 days' incubation at 38° the contents of each flask were washed into a 20 ml. graduated flask, and made up to volume with distilled water. Each solution was mixed thoroughly and its absorption spectrum determined.

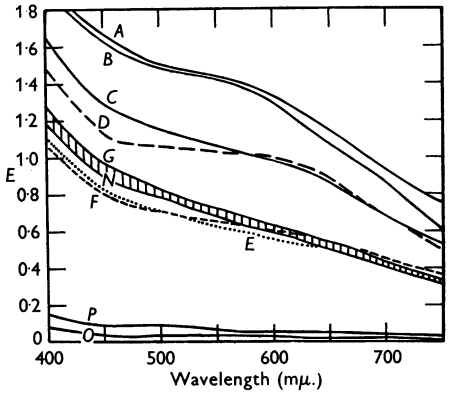

Fig. 3. Absorption spectra of melanins synthesized from 1 mg. tyrosine in the presence of 2 ml. of an enzyme preparation from cattle irises and ciliary bodies, a little Na_2CO_3 and various metals in the form of sulphate or chloride salts. The pH of each reaction mixture was approximately 6·8 and the total volume 4·1 ml. Solutions diluted to 20 ml. with twice distilled water prior to reading extinction coefficients. Metals added to the reaction mixture were as follows (pH values in brackets): *A*, 25 μg./ml. Zn (6·7); *B*, 20 μg./ml. Zn (6·6); *C*, 12·5 μg./ml. Zn (6·6); *D*, 25 μg./ml. Co (6·6); *E*, 5 μg./ml. Fe^{3+} (6·6); *F*, 5 μg./ml. Fe^{2+} (6·6); *G*, nil. (6·6); *H*, nil (6·6); *I*, 2·5 μg./ml. Cu (6·5); *J*, 25 μg./ml. Ca (6·6); *K*, 20 μg./ml. Ca (6·6); *L*, 25 μg./ml. Mg (6·5); *M*, 25 μg./ml. Ba (6·6); *N*, nil (6·5). Curves *G* and *N* (controls) form the limits of the shaded area; curves *H*–*M* (alphabetical priority indicates a higher position on the ordinate scale) are all included within this area. *O*, reaction mixture (before onset of melanin formation) diluted to 20 ml. with water; *P*, ditto + 25 μg./ml. Zn.

The experiments were of two kinds, (i) those in which the enzyme preparation was of type *A* (pH adjusted with sodium carbonate); (ii) those in which the enzyme preparation was of type *B* (stabilized at pH 6·8–7·0 with sodium citrate and sodium hydroxide). The results of the two groups differ from each other.

The absorption spectra of the various solutions of two experiments in the first group are given in Figs. 3 and 4 and of an experiment in the second group in Fig. 5.

Mason (1948) has shown that the absorption spectra of melanins enzymically produced from dopa vary slightly with the pH of the reaction mixture. The variation in the pH is not sufficient to

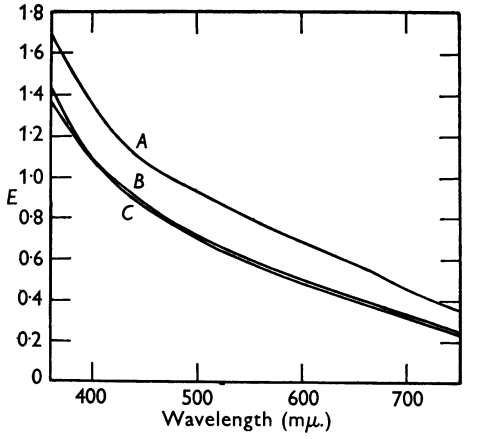

Fig. 4. Absorption spectra of melanins synthesized from 1 mg. tyrosine in the presence of 2 ml. of an enzyme preparation from cattle irises and ciliary bodies and a little Na_2CO_3. The pH of each reaction mixture was approximately 7·8, and the total volume 4·1 ml. The solutions were diluted to 20 ml. with twice-distilled water prior to reading extinction coefficients. Ca (25 μg./ml.) was added to the reaction mixture which produced solution *A*; *B* and *C* were controls containing no added metals.

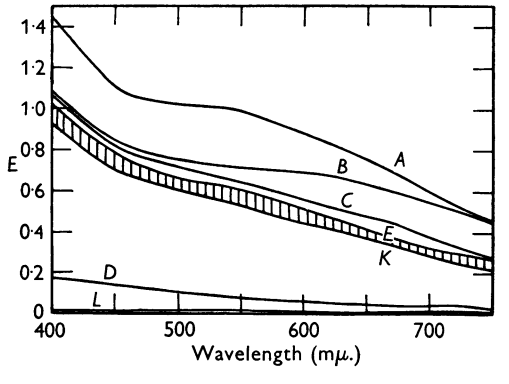

Fig. 5. Absorption spectra of melanins synthesized from 1 mg. tyrosine in the presence of 2 ml. of an enzyme preparation from cattle irises and ciliary bodies, buffered to about pH 7 with sodium citrate and NaOH, and various metals in the form of sulphate or chloride salts (except where otherwise stated). The total volume of each reaction mixture was 4·1 ml. Each solution was diluted to 20 ml. with twice-distilled water prior to reading extinction coefficients. Metals added to the reaction mixture were as follows (pH values in brackets): *A*, 25 μg./ml. Zn as $ZnCO_3$ suspension (7·4); *B*, 25 μg./ml. Fe^{2+} (7·1); *C*, 20 μg./ml. Zn (7·4); *D*, 12·5 μg./ml. Cu (7·2); *E*, 25 μg./ml. Zn (7·1); *F*, nil (7·0); *G*, 25 μg./ml. Ca (7·1); *H*, 25 μg./ml. Mg (7·1); *I*, 25 μg./ml. Ba (7·1); *J*, 25 μg./ml. Co (6·9); *K*, nil (7·0); Curves *E* and *K* form the limits of the shaded area. Curves *E*, *F*, *G*, *H*, *I* and *J* (alphabetical priority indicates a higher position on the ordinate scale) are included in the area. *L*, 100 μg. of Zn as $ZnCO_3$ suspension in 20 ml. water.

account for differences in absorption spectra such as those illustrated in Figs. 3 and 5.

Further experiments of type (i) showed that zinc added to the reaction mixture before the onset of melanin formation had a considerably greater effect in raising the absorption intensity of the melanin formed than did the same amount of zinc added 12 hr. before the solutions were removed from the incubator for dilution and measurement of light absorption.

In a further experiment of type (ii) it was shown that the addition of suspensions of barium carbonate, aluminium oxide, silicon dioxide and calcium carbonate to the reaction mixture, gave no increase in the final light absorption of the melanin solutions, and that zinc sulphate plus sodium carbonate had a greater effect than a zinc carbonate suspension containing the same quantity of zinc.

DISCUSSION

Many observations suggest that a number of metals may be associated with the presence of melanin pigmentation in animals. The evidence has not previously been assembled, and it is therefore necessary to review the literature briefly. The pertinent observations come under two heads: (i) those on the composition of pigmented tissues, and of melanin fractions from such tissues; (ii) those concerning melanin formation *in vitro*.

Evidence of an association of zinc with melanin pigmentation in the eyes of many vertebrates, and of a number of other metals occurring in melanin-protein fractions from the pigmented eye tissues, has been presented by Bowness *et al.* (1952) and Bowness & Morton (1952). Some findings of other workers such as the occurrence of barium in cattle choroids (Ramage & Sheldon, 1931) and the occurrence of high concentrations of copper in the ink of *Sepia officinalis* (Giuliani, 1938) were also mentioned.

Iron has many times been shown to be a constituent of melanin preparations. Sieber (1886) found it in melanins from the uveal tract of the eye, and from melanomas. Gortner (1911) found that ferric oxide was the chief constituent of the ash which made up 2–3 % of the dry weight of pigments from the black hair of rabbits, black feathers, and dark horse-hair. Waelsch (1932) recovered 1·9 % of the original dry weight as ash after incinerating choroid melanin, and showed that it contained iron. Flesch & Rothman (1945) isolated from red human hair a red, ferric-iron containing pigment, which had some of the properties of melanin. Nickerson (1946) showed that the white hair of rats and guinea pigs contained considerably less iron than red hair, and that white hair contained slightly less copper than black hair from the same animal. In this laboratory it has been shown that the white hair from rabbits (Fore, 1950; Bowness, 1951) and from rats (Bowness, 1951) gives rise to much less ash on incineration than black hair from the same animal.

The elements present in pathologically pigmented corneas have been compared with those present in normal corneas, but unfortunately the nature of the pigment which occurs in the pathological state was not investigated. Eckardt, Stolzar, Adam & Johnson (1943) give a list of the metals in normal human corneas, and in those of patients suffering from hepato-lenticular degeneration (Wilson's disease). In this condition a brownish pigment is deposited in the corneal membrane of Descemet. Na, Ca and Mg occur in the normal corneas, whereas Na, Ca, Mg, Zn, Cu, Fe, Al and Ag occur in those from the patients.

Complementary to the evidence obtained from analyses of melanin pigments and pigmented tissues, *in vitro* studies have indicated the influence of various metals on melanin formation. Gessard (1901) stated that the precipitation phase, at the end of the tyrosine-tyrosinase reaction, was influenced by various neutral salts. The alkaline-earth salts were much more active than the alkali-metal salts. Piettre (1911) confirmed that various salts aided the precipitation of melanin prepared by the use of animal tyrosinase. Durham (1905) found it expedient to add ferrous sulphate to the reaction mixture when she was testing various animal tissues for tyrosinase activity. Wager (1949) found that iron increased the colour of the pigment responsible for the stem-end blackening in the potato.

There is thus much evidence which suggests that a number of metals combine with, and perhaps influence the colour of, natural melanin complexes. Melanins can be formed, *in vitro*, from dopa under near-physiological conditions, in the absence of any metal, or (from tyrosinase) in the presence of only one, namely the copper in tyrosinase. It is therefore evident that melanins can be formed without any metallic combination.

However, Flesch (1949) has shown that though melanin can be formed by oxidizing dopa by molecular oxygen, this reaction is catalysed by the presence of Cu, Co, Ni, Mn, Pb and Fe, the magnitude of the effects increasing in that order. Zn had very little effect. In the present work it was found that Cu, Co, Fe^{2+} and Fe^{3+}, in that order, catalysed the oxidation of dopa to melanin by hydrogen peroxide. Zn, Ca and Mg had no effect, and Al and Ba ions inhibited the reaction slightly. When the same ions were added to preformed melanin a different effect was evident. The four cations which had a catalytic effect on the formation of melanin also caused an increase in the light absorption of the preformed melanin, but the magnitude of the effects on the absorption spectra of the melanin did not correspond with the catalytic effects.

Though it is possible that the small peak at 280 mμ. in the absorption spectrum of the dopa-melanin (Fig. 1) is due to unchanged dopa, and therefore that an increase in the light absorption on the addition of metals is due to formation of pigment from this residual dopa, this would not explain why the effects of both ferrous and ferric iron on the absorption curve are greater than that of cobalt, or the absorption above 580 mμ. of the solution containing ferrous iron greater than that of the solution containing copper. It seems probable, therefore, that the ferrous and ferric iron must form some sort of complex with the dopa-melanin itself. The fact that the absorption spectra of the solutions to which cobalt and copper were added (as well as those to which iron had been added) still showed inflexions at 280 mμ. due to dopa, indicates that the effect of even these two metals may not be explained by an additional formation of melanin. The absorption spectra thus imply that ferrous and ferric iron, and possibly copper and cobalt as well, can form complexes with dopa-melanin, but that zinc, calcium and barium do not form the same kind of complex.

The experiments on the formation of melanin from tyrosine in the presence of an enzyme solution extracted from cattle irises and ciliary bodies show that zinc and cobalt, calcium, and ferrous iron, can all, under different conditions, increase the light absorption of the final pigment formed by the reaction. Zinc and cobalt were effective in solutions containing carbonate at about pH 6·8, and calcium in solutions containing carbonate at pH 7·8 (approximately), but had no effect in solutions buffered with sodium hydroxide and sodium citrate, except in the case of zinc added as zinc carbonate. Ferrous iron was effective in the solutions buffered with sodium citrate and sodium hydroxide, but not in the solutions containing only carbonate. The complete inhibition of melanin formation in the presence of 12·5 μg. Cu/ml. reaction mixture recalls the work of Figge (1948), who showed that various reversibly oxidizable substances could enhance the rate of oxidation of dopa by atmospheric oxygen, but could completely inhibit the oxidation of tyrosine in the presence of tyrosinase.

Since no zinc is taken into non-ionic combination by melanin formed in the absence of protein, whereas some zinc combines with the protein in an extract from cattle irises and ciliary bodies, and much more is bound when melanin is formed from tyrosine in the presence of this protein, it is apparent that some complex between melanin and protein is operative in the binding of the extra zinc. The melanin-protein-zinc complex formed *in vitro* by this means may well be analogous to the melanin-protein fractions obtained from eye tissues, which were also found to contain zinc in non-ionic form. It is significant that if the melanin formed *in vitro* is assumed to combine with its own weight of protein, as was shown to be the case for the natural cattle melanin-protein complex (Bowness *et al.* 1952), then the amount of zinc bound to the synthetic complex is of the same order as that which is combined with the pigment material obtained from perch and frog eyes (Bowness & Morton, 1952), which contains the highest concentration of zinc yet found in biological material.

It is possible that the difference between the absorption curves of melanin formed *in vitro* in the presence of zinc, and in its absence, may be related to the difference between the absorption curves of melanin fractions obtained from frog and perch eyes (which contain very high concentrations of zinc) and those from cattle eyes (which contain very much less zinc).

The formation of complexes between melanin, protein and other metals has not been investigated in detail, but calcium, cobalt, and ferrous iron have been shown to affect the synthesis of melanin in the presence of protein, for they enhance the resulting light absorption. Interactions of copper, ferrous and ferric iron, and cobalt with dopa-melanin in the absence of protein have also been demonstrated. In general, the interactions of some metals with melanin, and some with melanin and protein, provide explanations for the occurrence of these metals in pigmented materials.

There are indications that melanins may be combined with proteins in very many biological situations. Henry (1935) showed that soluble melanin preparations give a fairly specific precipitin test with the serum of malaria patients. This shows that melanins can combine chemically with natural proteins. Adant (1932) showed that various natural melanins are antigenic, but that they lose their activity after treatment with hydrolysing agents which attack proteins. Greenstein, Turner & Jenrette (1940) showed that melanin was associated with protein in melanoma tissue; Serra (1946) and Stary & Richter (1938) showed that a melano-keratin complex occurs in hair. Melanin in these tissues could not be obtained free from protein by any enzymic treatment, and could only be purified by prolonged treatment with acid or alkali. Drastic treatment is considered necessary by most workers who have attempted to obtain melanin free from protein.

Nevertheless, chemically, protein and metallic constituents are not necessary parts of all melanin pigments; in the physiological state, however, they appear often to be associated. Some of the metals which occur in natural melanin complexes have been found to affect the light absorption properties of melanins formed *in vitro* from tyrosine. It is possible that such an effect can account for some of the differences in shade and intensity of natural

melanins. In this sense, therefore, those metals which appear to be frequent, if not universal, constituents of tissues containing melanin pigment, could be regarded as having a physiological function.

SUMMARY

1. The changes in the absorption spectrum obtained by adding various metals to a 3:4-dihydroxyphenyl-L-alanine- (dopa-)melanin solution have been investigated.

2. An enzyme preparation from cattle irises and ciliary bodies, which catalyses the oxidation of tyrosine to melanin, has been extracted and partially purified.

3. In solutions adjusted to pH 6·8 with sodium carbonate, zinc and cobalt were found to enhance the light absorption of melanin formed from tyrosine in the presence of the ocular enzyme extract. In solutions at pH 7·8 calcium acted similarly. In solutions buffered with sodium citrate and sodium hydroxide, ferrous iron enhanced the light absorption of the melanin formed.

4. Zinc was found not to combine at all with melanin formed in the absence of protein; it combines to some extent with the protein in the ocular enzyme extract, but is bound to a much greater extent when melanin is formed in the presence of this protein.

5. The possible existence of a physiological association between melanin, protein and various metals is discussed.

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Studies in Vitamin A

23. VITAMIN A AND ITS OCCURRENCE IN *AMBLYSTOMA TIGRINUM*

BY F. D. COLLINS, R. M. LOVE AND R. A. MORTON
Biochemistry Department, University of Liverpool

(*Received* 18 *July* 1952)

Vitamin A_2 has been generally regarded as characteristic of freshwater fishes in the same way as vitamin A_1 belongs to saltwater fishes and higher vertebrates. This view can easily be over-simplified; many saltwater fishes contain both vitamins although vitamin A_1 predominates.

Until recently, vitamin A_2 had only been found in fishes and in a few mammals whose diet consisted of freshwater fish, but in 1942 Wald discovered vitamin A_2 and porphyropsin in the newt *Triturus viridescens*. He later found (Wald, 1946) that vitamin A_2 and the porphyropsin visual system also occurred in the tadpoles of the bullfrog (*Rana catesbiana*). When metamorphosis began to take place, vitamin A_1 was detected, and the proportion of vitamin A_1 to vitamin A_2 increased as metamorphosis progressed, until in the adult frog all the vitamin A_2 had been replaced by vitamin A_1. Since