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The Role of Copper in Mammalian Pigmentation.

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Copper is an essential dietary factor for maintaining the color of fur in several species of mammals. Keil and Nelson[1] observed that black and piebald rats turned grey when fed on a diet deficient in copper. The original hair color could be restored within a few weeks by daily addition of 50 μg of copper to the diet. These observations were confirmed and extended to other species of mammals.[2-4] The depigmentation of copper-deficient rats and rabbits could not be cured by supplementing the diet with manganese, iron[4,5] or with vitamin B factors.[6] Copper was assumed to act as a catalyst of mammalian pigment formation[7] but there was no direct evidence to support this theory.

Experimental. The experiments which follow were carried out to elucidate the possible role of copper in the production of melanin. One set dealt with the effect of heavy metal catalysts and of inhibitors on melanin formation *in vitro*. In another series copper determinations were carried out on pigmented biological material.

The darkening of buffered solutions of dopa (1 - dihydroxyphenylalanine, Hoffmann La Roche) was measured colorimetrically. All vessels and test tubes were washed with a 1:1 mixture of concentrated HCl and HNO_3 and rinsed 5 times with both distilled and double-distilled water. To avoid introducing traces of heavy metals, barbiturate buffer of pH 7.4 was used throughout and all solutions were made with double-distilled water. Each test tube contained 1 ml of 5:10,000 dopa solution, 1 ml heavy metal salt solution in a 10^{-4} to 10^{-6} M final concentration and 3 ml of the buffer. The test tubes were kept in the incubator at 37° and readings were made in the Klett-Summerson photoelectric colorimeter with filter KS-42 at hourly intervals. Cysteine hydrochloride (2×10^{-3} to 10^{-4} M), phenylthiourea (10^{-4} M) and aqueous extracts of isolated human epidermis[8] were used as inhibitors.

Copper was determined in hair and tissues. The hair was clipped from the animals, washed 5 times with chloroform for several days and then with acetone, dried, weighed and ashed at 500°C. The tissues were defatted according to Greenstein[9] and ashed. The ash was heated with 1 drop of concentrated HNO_3, dissolved in 6 N HCl and the copper determined by the diethyldithiocarbamate method.[9] Iron was estimated by the 2-2′ bipyridine method[10] and manganese by the catalytic method of Kun.[11] All determinations were carried out in duplicate and only data agreeing within 10% were accepted.

The melanin used for copper analysis was prepared from Harding-Passey melanomas of mice by digesting the ground tumor tissue

* American Cancer Society Fellow, 1947-48.

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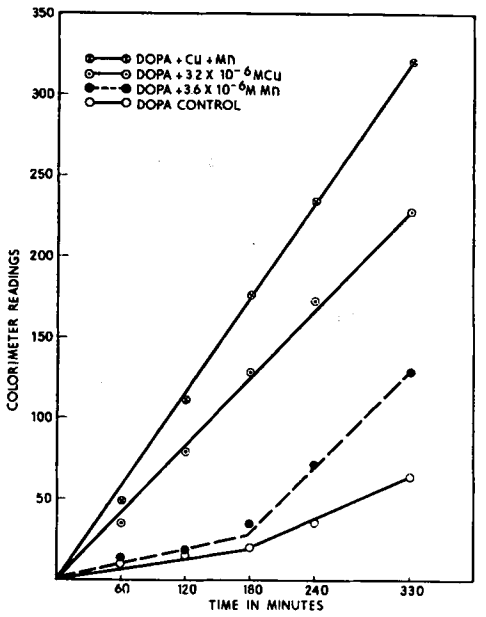

FIG. 1.
Catalytic effect of cupric and manganous ions on the autoxidation of dopa.

with pepsin-hydrochloric acid or with trypsin[12] for 4 weeks. The solution was changed at the end of 2 weeks. The digested tumor tissue was centrifuged and the supernatant fluid was dialyzed against double-distilled water for 7-10 days in the ice-box. The contents of the dialyzing bag were centrifuged, and the black precipitate was washed several times with double-distilled water and analyzed. In another series of experiments ground tumor tissue was kept overnight in 1% NaOH (5 ml/g wet tissue) at room temperature, then boiled for 30 minutes. The dissolved tissue was purified by dialysis, then dried for analysis.

Human epidermis from autopsy material was isolated by the heat method of Baumberger *et al.*,[13] or by treating the skin with 2 N KI solution.[14] Copper determinations were carried out on the defatted dried material.

Results. Catalytic activity of heavy metals on the autoxidation of dopa in vitro. The order of caralytic effect of the metals studied on the autoxidation of dopa was Cu>Co>Ni>Mn>Pb>Fe. Zn had very little effect, Mg and Hg none. The effect is additive (Fig. 1). In agreement with Bernheim[15] it was found that phenylthiourea which inhibits the tyrosine tyrosinase reaction *in vitro*[16,17] and pigmentation *in vivo*[18] had no effect on the autoxidation of dopa. In relatively high concentrations (10^{-4} M) it had only a slight inhibitory influence on the catalytic activity of cupric ion in the oxidation of dopa.

One mole of cupric ion was able to counteract the inhibition of the autoxidation of dopa produced by approximately 500 moles of cysteine (Fig. 2). Experiments with aqueous extracts of isolated epidermis confirmed the findings of Rothman and coworkers of an inhibitory factor in these extracts.[8] The factor was found to be heat stable and dialyzable and antagonized by cupric ion and by p-chloromercuribenzoic acid. This supports the previously advanced theory that the inhibition is due to sulfhydryl compounds.

Copper and iron determinations in hair. As suitable pigmented biological material, the hair of mottled rabbits, guinea pigs and rats

TABLE I.
Copper in μg/g in the Hair of Pigmented and White Areas of the Same Animal.

Animals	Copper content in μg/g hair	
8 rabbits	Grey or black: 35.4 (23.2-41.4)	White: 25.1 (9.3-35.8)
2 "	Light brown: 22.5 (22.2-22.8)	White: 20.6 (20.2-20.9)
5 guinea pigs	Black: 26.2 (9.0-46.0)	White: 15.5 (1.0-47.1)
2 " "	Black: 16.4 (13.0-19.8)	Red-brown: 8.7 (6.6-10.8)
3 " "	Red-brown: 26.5 (18.5-35.5)	White: 26.6 (18.3-36.3)
4 rats	Black: 29.1 (13.3-38.9)	White: 23.5 (13.9-31.6)

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TABLE II.
Copper in Human Epidermis in μg/g Dry Fat-free Tissue After Separation of Epidermis from Corium.

White epidermis		Corium	Negro epidermis		Corium
No. 1	73.5	35.4	No. 1	50.2	15.2
No. 2	53.0	39.6	No. 2	53.5	24.4
No. 3	88.9	28.7	No. 3	51.9	11.1
No. 4	29.7	12.0	No. 4	49.4	6.2
No. 5	56.3	18.1			
No. 6	142.7	33.0			
No. 7	186.6	31.5			
No. 8	180.2	—			
No. 9	139.4	—			

TABLE III.
Copper Determinations in Harding-Passey Melanomas and in Melanin Prepared from the Tumor.

No.	Wet wt of tumor. g	Water content, %	Cu in μg/g dry fat-free tumor tissue	Cu in μg/g melanin
1	7.10	79.7	11.5	—
2	6.75	79.4	13.5	—
3	5.26	79.0	12.5	50.4
4	5.20	—	26.2	337.9
5	4.60	79.7	15.1	—
6	4.28	78.8	17.5	163.0
7	2.45	81.3	22.4	—
8	1.95	78.5	26.5	—
9	0.52	—	59.2	294.0

was chosen for copper determinations. By comparing white and colored hair from the same animal, individual variations in the copper content due to age, strain, and diet of the animals[19] were excluded. The results are presented in Table I.

The black or grey hair of 6 of the 8 rabbits, 6 of the 7 guinea pigs, and 2 of the 4 rats tested contained significantly more copper than the white or red brown hair of the same animals. No difference was found in the copper content of white and brown hair.

Determinations carried out on guinea pig hair showed that the iron content of the red-brown hair was considerably higher than that of the white hair of the same animal. The mean values in 6 animals were 35.4 μg/g (25.0–46.1) for red-brown hair and 15.4 μg/g (4.5–23.2) for white.

Copper determinations in isolated human epidermis. Isolated human epidermis obtained from autopsy material showed the same large individual variations in copper content as had the hair of animals. Preliminary experiments with Negro epidermis indicated a copper content in the same range as that found in white specimens, but not enough experimental material was available to allow making definite conclusions. The copper content of the epidermis invariably was higher than that of the corium. (Table II).

Copper determinations in Harding-Passey mouse melanomas. The tumor tested has been carried in this laboratory for one year in mice of the dba strain. Copper determinations were made on such tumors and on the kidneys and spleens in normal and tumor bearing animals. In the same animal the copper content of melanomas was below that of the kidneys (71.3 μg/g dry weight) and spleens (52 μg/g). Larger and older tumors had a lower copper content than recently implanted ones and all tumors had a relatively high water content.

The melanin pigment prepared from the melanomas by boiling with NaOH, contained 4 to 13 times more copper than the tumor. (Table III) In the amounts used in the preparation, the NaOH could have accounted for only about 5% of the copper found in the

[19] Cunningham, I. J., *Biochem. J.*, 1931, **25**, 1267.

melanin, a percentage within the experimental error of the copper determination. On the other hand, the melanin pigment prepared by peptic and tryptic digestion yielded a copper content 20 to 95 times higher than the tumor tissue. The difference may have been due to more complete hydrolysis of the tumor tissue, but since both the pepsin and trypsin used had relatively high copper contents, the possibility could not be excluded that some copper in the pigment prepared by digestion was of extraneous origin. These data are therefore not presented. On the other hand, the manganese content of the tumor (57.3 μg/100 g dry fat free tissue) was practically identical with that of the pigment (63.2 μg/100 g) derived from it. Values obtained with liver and kidney were in the same range.

Discussion. The catalytic influence of cupric ions on the autoxidation of dopa has been described by several authors.[7,19] No comparative experiments with other heavy metallic salts have been reported. Sarata[7] observed that copper sulfate exerted a catalytic effect only in a certain range of concentration. In this laboratory the apparent inhibition by relatively high concentrations of copper sulfate, described by Sarata, was found to be due to the use in his experiments of unbuffered solutions; in relatively large amounts, copper sulfate lowered the pH below the neutral range. In some of our experiments, involving the use of buffered solutions, copper sulfate in concentrations which Sarata had found to be inhibitory (5 μg/ml) showed a more powerful catalytic action than in lower concentrations. Copper determinations on the hair of mammals have been made by several authors. In two papers its concentration was reported to be higher in black hair than in white hair from the same animal,[7,19] while in others no such difference was found.[20,21] However, the determinations were on a very limited number of animals. Melanogenesis takes place in the germinative epithelial layer of the hair and even if copper is involved, it may not ascend with the pigment in the growing hair. The higher iron values in red-brown guinea pig hair than in white hair are of interest in view of the fact that an iron-containing red pigment has been isolated from human red hair.[22] The literature has no data on the copper content of isolated human epidermis. Relative values for whole skin, expressed in relative intensities of spectral lines, showed the same large individual variations as observed in our experiments.[23] In bovine epidermis, Cunningham[19] found much larger values than in the dermis of the same animal. He suggested that the skin was an important organ for copper excretion. Since the pigment constitutes such a small fraction of the entire epidermis, a difference in the copper content of pigmented and non-pigmented skin may not be detectable. This would explain the relatively low copper values in the epidermis of the Negro. The low copper values of melanotic tumors agree with the data of other authors.[19,24,25] However, the finding

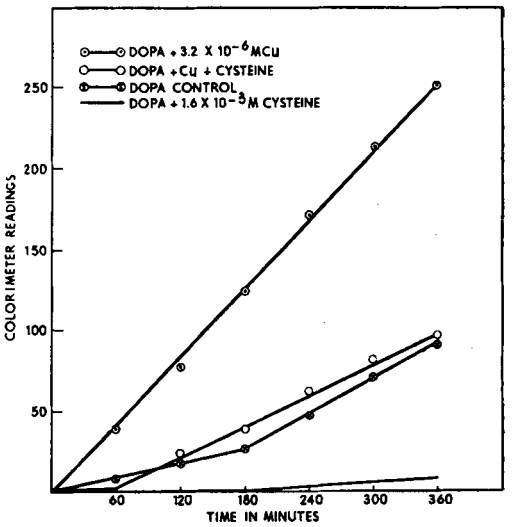

FIG. 2.

Suppression, by cupric ion, of the inhibitory effect of cysteine hydrochloride on the autoxidation of dopa.

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that the isolated pigmented part of the tumor is strikingly high in copper concentration strongly favors the theory that copper acts as a local catalyst in pigment formation, probably as part of an oxidative enzyme, possibly tyrosinase which has recently been demonstrated in melanotic tumors of mice.[26,27] Mammalian tyrosinase contains significant amounts of copper and can be inhibited by substances which combine with this metal.[28] Copper apparently also forms a metallo-organic complex with dopa[29] and may remain attached to the melanin molecule. In this connection it is of interest to note that the ash of octopus ink contains as much as 2% copper.[30]

We advance the theory that copper may promote pigmentation, not only by direct action on the substrate, but also indirectly by oxidizing sulfhydryl groups which inhibit pigmentation *in vitro*,[31] and probably also *in vivo*.[8,32,33] Greenstein *et al.* have indicated that melanin in mouse melanomas is attached to the rest of the pseudoglobulin molecule in the vicinity of sulfur containing amino acids.[12] Our finding that copper, too, is closely linked to the pigment suggests an interaction between copper and sulfhydryl compounds.

Summary 1. Autoxidation of dopa *in vitro* was more strongly catalyzed by cupric ions than by any of the other heavy metallic salts investigated. The order of catalytic activity was Cu>Co>Ni>Mn>Pb>Fe.

2. Black and grey hair of rabbits, guinea pigs and rats generally, but not always, contained more copper than white hair of the same individual. In guinea pigs, red-brown hair contained more iron than white hair.

3. Analysis of the separated layers of human skin showed a considerably higher copper content in the epidermis than in the corium.

4. Melanin from mouse melanomas had a copper content 4 to 13 times higher than the tumor from which it was prepared.

5. The theory is advanced that copper plays a role as a local catalyst in mammalian pigmentation.

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An Antidiuretic Substance in the Blood of Normal and Adrenalectomized Rats.*

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In adrenal insufficiency the diuretic response to water is greatly reduced or absent (reviewed by[1]). An attempt was made to see if this was due to the accumulation of anti-

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