THE MELANINS. I. STUDIES OF THE HAIR PIGMENTS OF THE GUINEA PIG[1]

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INTRODUCTION

ALTHOUGH the chemical and physical characteristics of the melanin pigments have been widely studied, the differences which give rise to the many colors and intensities have not as yet been satisfactorily determined. Geneticists have been handicapped by this lack of knowledge when they attempted to provide a logical chemical background for known hereditary relationships from which to obtain important knowledge of genic action. For example, the chemical relationships between the melanins of different colors are not known, and it is not certain that any consistent chemical difference distinguishes them. It will be the purpose of the present work to show that the melanins from guinea pig hair may be chemically distinguished by means of the spectrophotometric technique.

RAPER (1927) has investigated the tyrosinase-tyrosine reaction, long considered the most likely source of melanin, and has found that black melanin is formed by the oxidation and increase in molecular size of certain products from the reddish-colored quinone of 5, 6-dihydroxydihydroindole-2-carboxylic acid. HAEHN (1921) also considers that an increase in particle size yields the black melanin, with intermediate colors—brownish red, violet brown, dark brown, etc., due to intermediate particle sizes. All these colors are actually found in the hair of mammalia but have not been proven to be related as have the differently colored products of the tyrosine-tyrosinase reaction. However, all the pigments in this study of guinea pig hair are referred to as melanins. That differences in particle size of a single chemical substance may be responsible for this range in color often has been suggested (JANKOWSKY 1935).

Probably the earliest spectrophotometric work with melanins was that of GALLERANI (1923) and others in Italy. They attempted to show that melanins are derived from pyrrolic compounds and not from tyrosine. DANIEL (1938) investigated the absorption curves of black, several forms of dilute black, and chocolate mouse melanins and demonstrated that these are similar except for concentration differences. Moreover, she found that the curves she obtained closely resembled that of black horse hair melanin, as reported by ZWICKY and ALMASY (1935). She concluded that the pigments are probably chemically identical. ZWICKY and ALMASY (1935) also determined the curve for red horse hair melanin and declared that the red and black pigments are spectroscopically indistinguishable. From examination of their data, however, it does not

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seem possible to make the two curves coincide by multiplying one or the other by any concentration factor, as should be the case if the two curves were given by the same substance. ARNOW (1938) has previously expressed the opinion that the red and black curves of ZWICKY and ALMASY appear dissimilar. Statistical methods for comparing the curves given by solutions of the different melanins have not been used by any of the above authors.

METHODS

Solutions for spectrophotometric comparisons were made up in the following manner. After defatting by extraction with CCl_4 for two or more hours in a Soxhlet apparatus, the hair (0.125 gm sample) was boiled in 1 percent NaOH for approximately two hours. Water was added as needed to keep the volume approximately constant. After cooling, the solutions were filtered, made to 100 cc volume, and a portion used in a Bausch and Lomb spectrophotometer to determine the absorption curve. The setting of the spectrophotometer was checked to the sodium D lines during the course of the experiments. Absorption was determined at four wave lengths, since it was found that these sufficiently described the curves between 470 mμ and 600 mμ. The averages of the readings (usually ten in number) at each point, and often made by two individuals independently, determined the curve.

During filtering, some pigment was usually retained on the filter paper. The amount so held apparently depended on variations in heating, the amount of adsorption by the keratin residue, and the kind of pigment involved. Black melanin solutions left the filter paper quite black, while red melanin was only slightly retained under similar conditions. DANIEL (1938) has noted that the shape of the curves is not affected by this filtering out of part of the pigment, since all the pigment of a given sample when dissolved gave the same curve as the first fraction. The curves given by solutions of black melanin from guinea pig hair, however, were altered by five hours boiling time; therefore, the period of extraction with the boiling alkali was limited to two hours or less.

TABLE 1

Description and genetic composition of melanins.

NUMBER	GUINEA PIG NO.	DESCRIPTION	GENETIC COMPOSITION
1	W908.4	intense black *E*	*P Sm Ee C aaBb didi*
2	W23.1	intense black e^p	*P Sm* e^p *C aaBB didi*
3	V200.1	c^r intense black	*P Sm* e^p c^rc^r *aaB*
4	V876.4	c^r dilute black	*P Sm* e^pe c^rc^r *aaBB*
5	X91.1	intense chocolate	*P Smsm E-C-aabb didi*
6	S756.2	cherry red *bb*	*P Sm eeC bb*
7	U218.4	cherry red *B*	*P Sm eeC Bb*
8	W311.1	albino	c^ac^a
9	—	dopa-melanin	—
10	—	intense black human hair (Caucasian)	—

The description by which a particular melanin is designated is presented in column three, table 1; in column four is shown the known genetic composition. The first two melanins are both black, but they differ in that the second comes from an animal having red spots (due to e^p) and the first from one that is entirely black (due to *E*). One would expect these two black melanins to behave similarly when compared spectrophotometrically. Most black and chocolate guinea pigs have hair less intensely pigmented at the base than distally; however, the *di* gene carried by melanins one, two, and five causes the hair to be almost uniformly pigmented. Melanins three and four differ from one and two in carrying gene c^r in place of *C*, and for this reason such hair appears less intensely black. Of the two, number four is definitely more dilute in appearance than three, although the inheritance of the genes responsible for this difference has not as yet been determined. The black guinea pig melanins might be ranged, then, in order of decreasing intensity of the blackness of the hair: 1 and $2 > 3 > 4$. Melanin five corresponds to one and two in intensity, but since it carries gene *b* for chocolate instead of *B* for black, it is intense chocolate in color. Numbers six and seven also differ only in that one carries *b*, the other *B*, but since these animals are also *ee*, which produces red, the chocolate pigment in number six and the black pigment in number seven are restricted to the skin of the ears, eyelids, nose, etc., leaving the hair entirely red. Cherry red is a term which distinguishes the dark red characterizing these animals and others of "show" type from the lighter red ordinarily found in laboratory stock. The albino, number eight, probably contains no melanin and was included in order to have a check on the effect of keratin degradation products. Dopa-melanin, number nine, is the designation given the solution resulting when 1 gm dopa (3, 4-dihydroxyphenylalanine) in 1 liter of water stood for approximately a year stoppered and in a paper cover, during which time it had oxidized to a very dense black solution with the formation of a small precipitate. The tenth melanin was obtained by dissolving samples of very intense black Caucasian human hair.

RESULTS AND DISCUSSION

DANIEL (1938) and SPIEGEL-ADOLF (1937) have observed that when the logarithm of optical density of melanin solutions is plotted against wave length, apparently linear curves result (between 250 mμ and 670 mμ). In the present experiments this method of graphing was adopted, but in addition, statistical methods of comparing the curves were used in order to obtain a more accurate measure of slight curve differences. A straight line may be fitted to the experimental points by the method of least squares, and this method gives a value, the regression coefficient, which expresses mathematically the slope of the calculated best line. For each sample of melanin, the logarithms of the optical densities at the four wave lengths were used and the regression coefficient calculated. The average regression coefficient and the number of samples for each melanin are given in table 2.

Curves calculated by the above method have been plotted in figure 1 for representative samples of several of the melanins. In order to compare these

TABLE 2

Average regression coefficients of log optical density against wave length for alkaline solutions of various melanins.

NO.	DESCRIPTION	SOLUTIONS PREPARED BY 2 HOURS' BOILING		SOLUTIONS PREPARED BY 2 WEEKS' EXTRACTION OF HAIR AT ROOM TEMPERATURE	
		NO. OF SAMPLES	AV. REGRESSION COEFFICIENT	NO. OF SAMPLES	REGRESSION COEFFICIENT
1	intense black *E*	10	−.020	1	−.042
2	intense black e^p	3	−.020	1	−.040
3	c^r intense black	6	−.025	1	−.040
4	c^r dilute black	2	−.033	1	−.040
5	intense chocolate	2	−.029		
6	cherry red *bb*	6	−.060	1	−.069
7	cherry red *B*	2	−.068		
8	albino	4	−.047		
9	dopa melanin	3	−.019		
10	intense black human hair	2	−.016		

and DANIEL's data, her curve for heterozygous Chinchilla mouse is also given. Genetically, this melanin of hers corresponds to c^r black guinea pig melanin and would be expected to have a similar spectrophotometric curve.

Regression equations for the curves of figure 1 are as follows:

intense black (*E*)	E = 1.018 − .019X
c^r intense black	E = 1.175 − .025X
cherry red (*bb*)	E = 2.734 − .059X
albino	E = 1.284 − .048X

The regression coefficient is the number preceding the X and has been calculated as the change in logarithm of optical density per change of 10 mμ in wave length. The negative sign indicates the inverse relationship. The curves appear linear in the range investigated, and there is reason to believe that this relationship holds for melanins free from protein in the ultra violet (SPIEGEL-ADOLF 1937).

The two black guinea pig melanin curves (*C* and c^r) and the black (Chinchilla) mouse melanin curve appear to have approximately the same slope in figure 1. Since that of the latter is about −.030, two curves whose regression coefficients differ as much as .011 are not readily observed to be different in slope by inspection of such a figure. Such a degree of similarity between two curves has been taken by previous workers to indicate that the melanins involved are spectrophotometrically indistinguishable and probably chemically alike. Within the above limits, it is evident from figure 1 that black guinea pig melanins give curves like black melanins from mouse hair and from horse hair. However, as will be shown later, the slight difference between these two black guinea pig curves, .005 in amount, is statistically significant. It follows

that a statistical treatment is more likely than visual inspection to reflect the differences which do occur. The curve for red guinea pig melanin, however, may readily be distinguished statistically from those of any of the black melanins. It is evident even from inspection that no shifting of the red curve

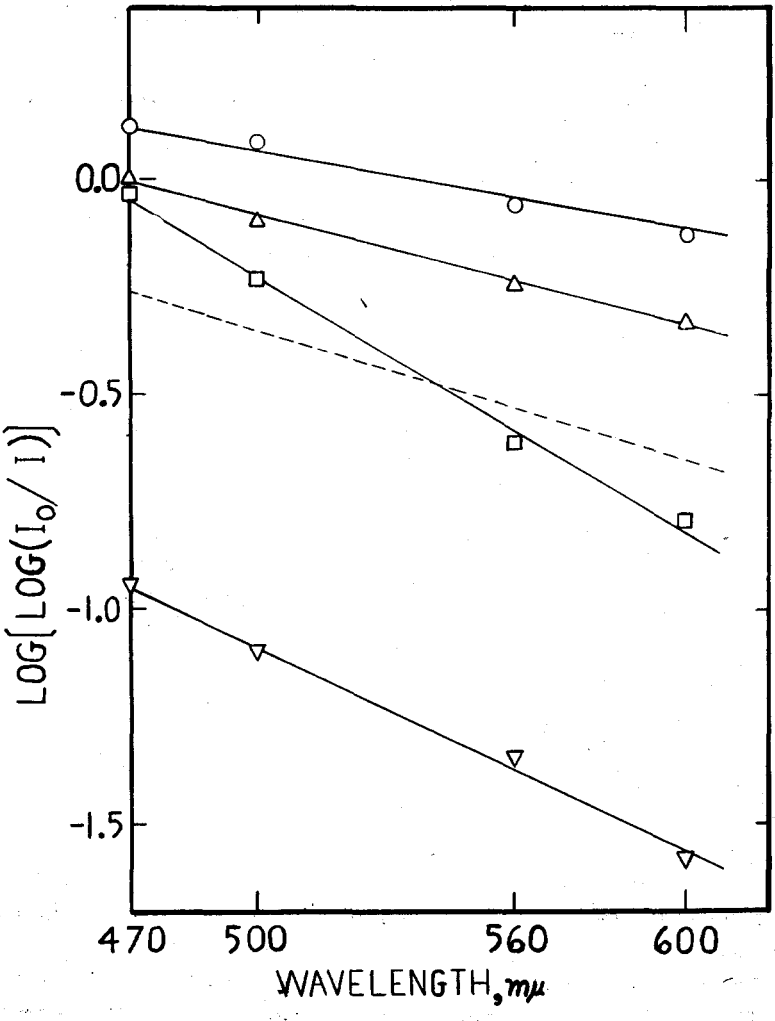

FIGURE 1.—Absorption curves of alkaline melanin solutions, log optical density (log [log (I_0/I)]) plotted against wave length. Guinea pig melanins: [*o*, intense black (*E*)], (Δ, c^r intense black), [□, cherry red (*bb*)], (▽, albino), [- - - - - -, heterozygous chinchilla mouse melanin, after DANIEL (1938)].

up and down by use of concentration factors (which does not change the slope of the curve) can make it coincide with any of the black curves. ZWICKY and ALMASY'S statement that black and red melanins may not be distinguished spectroscopically does not hold for guinea pig melanins. This evidence also would not support an explanation that the fundamental difference between

hairs of different colors depends entirely on the amount of pigment present. RUSSELL'S results (1939) seem to lead to the same conclusion, for she found that intense red guinea pig hair required more $KMnO_4$ to oxidize the pigment than did an equal weight of the extremely dilute black and chocolate hairs. EINSELE (1937) has suggested that qualitative differences may exist even between black and chocolate melanins, for equal weights of pigment from mice of different genotypes dissolved at the same concentration gave different color intensities in a colorimeter. In our experiment the regression coefficient for intense chocolate guinea pig melanin falls between those of the two c^r black melanins. Consequently, some physical differences not reflected by the spectrophotometric method must differentiate chocolate from black melanin.

The other curve in figure 1, albino, has been used by ZWICKY and ALMASY (1935) as a correction for the absorption of keratin degradation products. They subtracted this correction from the pigment curves. This procedure has not been followed here because the results are only slightly changed, and the albino curve has been found to be subject to considerable experimental error.

The extreme range between regression coefficients for the same melanin was .013 and occurred between two samples of cherry red *bb*. Since the other five samples were grouped around −.062, it is not unlikely that the low sample (−.050) was in error; nevertheless, all six are averaged together to give the regression coefficient −.060 for cherry red *bb* melanin. The ten samples of intense black (*E*) melanin ranged from −.017 to −.024, the next largest spread in values for a given melanin. These averaged −.020, and the variation can be attributed to experimental error. When the regression coefficients for c^r intense black and cherry red *bb* were compared statistically, it was found that the probability of their being random measurements of the same population regression coefficient is far less than one in a thousand ($t=25$, 0.1 percent level $=3.5$). Statistically highly significant differences may also be found among the regression coefficients of the black melanins (for intense black (*E*) and c^r intense black, $t=9$, 1 percent level $=2.7$). This may confirm EINSELE'S evidence for qualitative differences among different black and chocolate melanins, but the number of samples in all cases is small. The c^r dilute black curve is significantly different from the intense black (*E*) curve and, moreover, bears the same relation to it that the dilute black curve of DANIEL (1938) bears to her intense black mouse melanin curve. As DANIEL stated, the differences between the two appear small when the plotted curves are examined. However, the use of regression coefficients may indicate whether such small differences are consistent or without significance. DANIEL has further suggested that the curves may represent scattering and not true absorption. If this is the case, further study of melanin curves might lead to information of the relative particle sizes involved. Even if melanin solutions are not true solutions, conclusions from spectrometric observations are probably valid, since the Lambert-Beer Law has been shown to be generally true for hydrophobic colloids and in particular for solutions of melanins (See SPIEGEL-ADOLF 1937).

ZWICKY and ALMASY (1935) state that their solutions, obtained by extrac-

tion of the hair for two weeks at room temperature, did not alter in optical properties on long standing. Others found, however, that solutions prepared by boiling were apparently bleached by exposure to light or ultra-violet light or on further boiling (DANIEL 1938; EINSELE 1937). DANIEL (1938) has presented curves from samples of hair boiled 30 minutes and 165 minutes to show that the slope is unchanged although the apparent concentration decreases. In the present experiments not only were changes found in the apparent concentration of pigment as measured by the height of the spectrophotometric curve, but also the slope of the curve was altered on standing.

Table 3 illustrates these changes for intense black (*E*) melanin. The first line records the average optical density of two samples at 470 mμ at the time intervals indicated, while the second line gives the average regression coefficient for the two curves. The values at 120 days are from a different sample of the same melanin. Red melanin solutions in general changed much less rapidly but must be further studied before drawing definite conclusions. The average regression coefficient of two samples of red melanin changed from −.062 to −.069 after 18 days.

TABLE 3

Changes in optical density and regression coefficient of intense black (E) melanin solutions with time in 1 percent NaOH.

DAYS	0	1	2	4	7	11	18	120*
Optical density at 470 mμ	1.53	1.23	1.18	1.02	0.91	0.82	0.74	0.23
Regression coefficient	−.022	−.025	−.028	−.032	−.033	−.036	−.039	−.048

* Readings at 120 days made on a different sample of the same melanin.

Using ZWICKY and ALMASY'S method of extracting the hair two weeks with alkali, solutions were prepared which gave the regression coefficients in the last column of table 2. These values check quite well with those of table 3 for between 11 and 18 days; so it may be concluded that the changes which take place in the boiled solutions on standing also occur when ZWICKY and ALMASY'S method is employed. The greater similarity of the curves after 14 days may explain in part why ZWICKY and ALMASY decided the curves for red and black melanin are alike.

The change in slope of the curve of the black melanin solution on standing obviously brings it nearer that of the red melanin curve. ARNOW (1938) oxidized a solution of dopa melanin in air to a reddish solution having the same spectrophotometric curve as an extract of red human hair. Thus he concluded that red melanin is an oxidation product of black melanin. A comparison of ARNOW'S red melanin curves with those of red guinea pig melanin shows that the latter are definitely more steep. The former would have a regression coefficient of approximately −.04 while that of red guinea pig melanin is −.06. A sample of dopa melanin oxidized in this laboratory by bubbling air through it for two days in 1 percent NaOH changed its regression coefficients from

$-.021$ to $-.039$, but seemed to reach an equilibrium at that point. Treatment of this product with H_2O_2 produced a yellow solution with a regression coefficient of $-.062$. ARNOW's conclusion that red melanin is an oxidation product of black melanin apparently holds for guinea pig melanins, although red guinea pig melanin seems to have a definitely steeper curve than other red melanins yet reported.

A comprehensive theory of the processes involved in the melanin pigmentation of mammals has been proposed by WRIGHT (1917, 1927) and related to the known color genes in guinea pigs. His Substance I is determined by the *C* locus and is necessary for any pigment production. If Substance I is present in a concentration corresponding to c^r or a higher allele, black or chocolate pigment may be formed if Substance II determined by gene *E* is present; or red pigment may be formed if Substance I is present in a concentration corresponding to c^d or a higher allele, and Substance II is absent. The effects of other genes and conditions are also included in WRIGHT's hypothesis. Considerable investigation will be required to determine the precise effect of each gene in definite physiochemical terms, but some conclusions may be drawn from the present experiments.

If we accept the hypothesis that red melanin apparently differs chemically from black by being more highly oxidized, and since it differs genetically by having the constitution *ee* instead of *E*, it seems clear that the *E* gene determines the presence (1) of a less efficient oxidizer than *ee*, or (2) of an inhibitor of the oxidation process, or (3) of some other condition unfavorable to the oxidation of melanogen past the black or chocolate stage. Whichever alternative is true is represented in WRIGHT's scheme by Substance II. Gene *E* may then as well produce the absence of some agent in the oxidation process (or a lower concentration than *ee*) as the presence of a "Substance II."

The *B* gene produces an effect (in the hair) only in the presence of both Substances I and II, in which case it produces black pigment in contrast to true chocolate pigment produced by *bb*. The nature of the difference in action of *B* and *b* is not known, but the present work shows it is not so much a chemical difference, which should be detectable with the spectrophotometer, as a physical state, such as density or particle size of the pigments involved.

BOGART and IBSEN (1937) and IBSEN and BOGART (1939) assumed that when black hair was bleached with H_2O_2, black melanin became colorless and allowed red pigment to show. Since the present results have shown that the oxidation of black melanin in solution either by air by by H_2O_2 produces a reddish solution, another possible interpretation is that treatment of black hair with H_2O_2 changes the black pigment to red.

SUMMARY

The use of regression coefficients for log optical density plotted against wave length is proposed as a means of comparing the absorption curves of melanin pigments. By this method red and black guinea pig melanins have been shown to be easily distinguishable.

Some evidence is found that qualitative differences may exist between dif-

ferent black guinea pig melanins. However, their curves are similar to those of black melanins from mice and from horses, and also to that of dopa melanin.

Solutions of black melanin prepared by boiling hair two hours in 1 percent NaOH do not give the same curve after standing 24 hours; the resulting curve decreases in height and its slope shifts toward that characteristic of red melanin. Air oxidation is presumed to be the cause.

The regression coefficient for intense chocolate melanin falls within the range of those for different dilute black melanins; hence it is concluded that chocolate and black melanins are chemically alike and that the *B* locus probably determines some difference in physical state of the pigment present.

Additional evidence that red melanin is an oxidation product of black melanin is presented, and this difference is related to the *E* locus (in guinea pigs) in terms of WRIGHT's hypothesis regarding melanin pigmentation.

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