ENZYMATIC STUDIES OF THE PHYSIOLOGICAL GENETICS OF GUINEA PIG COAT COLORATION. I. OXYGEN CONSUMPTION STUDIES[1, 2]

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THE GENETICS of coat color pigmentation in the guinea pig have been extensively analyzed by WRIGHT (1916, 1917, 1925, 1927). These studies have been supplemented by various attempts to discover the underlying inherited physiological processes, either by quantitative determinations of pigmented end product (E. S. RUSSELL 1939; HEIDENTHAL 1940; WRIGHT and BRADDOCK 1949; WRIGHT 1949) or by assays for enzyme (dopa oxidase) activity in the skins of a wide variety of genotypes (W. L. RUSSELL 1939; GINSBURG 1944). A detailed consideration of these different types of studies is contained in WRIGHT's review (1942). The study reported here represents an additional attempt to ascertain inherited melanin-forming enzyme variations in guinea pig skin, and some of these results have already been summarized (FOSTER 1952b).

W. L. RUSSELL's histochemical studies with incubated frozen skin sections involved visual grading of degree of blackening both in hair bulb and in basal epidermal melanocytes. His assays for dopa oxidase activity indicated a strong correlation between the intensity of the hair bulb reaction (but not that of the basal epidermal melanocytes) and the amount of natural yellow pigment (phaeomelanin) controlled by the genotype. A similar correlation was reported by GINSBURG, who performed turbidimetric assays for dopa oxidase activity in fresh and stored ("delayed") skin extracts (1944). Our own results, while in many respects confirming those of RUSSELL and GINSBURG, tend on the whole, but with important exceptions, to indicate a correlation between genetically controlled amount of dark pigment (eumelanin) and tyrosinase or dopa oxidase activity. However, as will be discussed later, at least some of the major apparent discrepancies between reported results can be reconciled on the basis of differing materials and methods.

MATERIALS AND METHODS

Materials

The foetuses used in this study came from the stocks of PROFESSOR SEWALL WRIGHT, and the genotypes were determined by us on the basis of the foetal phenotypes and of the genotypic possibilities of each specific mating as determined by

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PROFESSOR WRIGHT'S examination of breeding records and the phenotypes of each pair bred. The major coat color allelic series are as follows:

1. *E* (predisposition for dark pigmentation), *e* (predisposition for yellow pigmentation), e^v (dark and yellow spotted).
2. *A* (agouti or yellow banded), *a* (solid dark color hair).
3. *B* (black pigment), *b* (brown pigment).
4. Albino series alleles; *C*, c^k, c^r, c^d, c^a. (Combinations lacking gene *C* show different degrees of reduction of dark and yellow pigments).
5. *F*, *f* (*ff* reduces amount of yellow pigment only, in presence of *P*; appreciable reduction of dark pigment only in association with *pp*).
6. *P*, *p* (pink-eyed dilution factor; *pp* always dilutes dark pigment, never yellow). Nearly complete absence of dark pigment in the double recessive, *ffpp* (pale cream phenotype). On a yellow (*ee*) background, however, *ffP-* is equivalent to *ffpp*.
7. *S*, *s* (*ss* gives white spotting).

The various genotypes studied by us were as follows:

Black (*E-* or e^v- *aaBCF* or *ffP*)
Black agouti (*EABCFP*)
Brown (*EaabbcF* or *ffP*)
Pale brown (*EaabbCFfpp*)
Pale sepia (*EaaBCFpp*)
"Red", most intense of yellow series (*ee——CFP*)
Pale cream (*E—bbC–ffpp*)
Lower albino series alleles, i.e.;
  Black (*EaaBc^{k}c^{r}FFP*)
  Dark sepia (*EaaBc^{r}c^{r}FFP*)
  Light sepia (*EaaBc^{r}c^{a}FFP*)
  Pale sepia (e^ve^v*aaBc^{k}c^{k}pp*)
  Albino (—c^ac^a—) or nearly white (*eec^{d}c^a*)

Methods

A Warburg constant volume respirometer was used to measure oxygen consumption as a measure of the enzymatic oxidation of tyrosine or dopa. The enzyme preparations were simply dorsal skins ground to a fine powder while frozen, as previously described (FOSTER 1951, 1952a). Because of the difficulty in demonstrating enzymatic activity in skins after birth, the dorsal skins of approximately 50 day old foetuses were used. At this foetal age (the date of the dam's previous litter taken as the date of conception) pigmentation is intense, the hairs have already emerged from the skin and the skin itself is ground in the frozen condition without much difficulty. As an additional check against gross errors in estimating foetal age the crown to rump length of each foetus was determined. This length varied between 7.5 and 10 cm for foetuses ranging in estimated age from 50 to 54 days. As an additional check on the classification of the foetal genotypes in segregating litters, a small sample of skin from each foetus was fixed in 1:10 formol-alcohol (½ hour), dehydrated in absolute alcohol (½ hour), cleared in benzene (at least one hour) and mounted in Permount (Fisher Scientific Co.). Skin homogenates not used on the day of preparation were stored at −20°C, and such homogenates stored as long as 8 months retained their characteristic

activities. In preparation for a Warburg run, homogenized dorsal skin from a single foetus was suspended in a measured amount of pyrex redistilled water and aliquots were pipetted into Warburg reaction vessels. The amount of skin per ml aliquot ranged in various experiments from $\frac{1}{20}$ to $\frac{1}{10}$ of a dorsal foetal skin.

The pyrex reaction vessels had a capacity of approximately 10 ml. The center wells contained 0.2 ml of 20% KOH, the main part of each vessel contained 1 ml of skin suspension, while the side arm contained 0.5 ml of M/10 phosphate buffer, pH 6.8, or substrate dissolved in an equal volume of buffer. The substrate solution consisted of L-tyrosine (0.5 mg/ml). Occasionally L-dopa (0.5 or 1.0 mg/ml) was used as exogenous substrate to check the results with tyrosine. When the enhancing agent, iodoacetamide, was used, it was dissolved in the buffer (with or without substrate) at a concentration of 6 mg/ml.

After temperature equilibration at 38°C (10–15 minutes), side arm contents were tipped into the flasks (*zero time*).

Endogenous oxygen uptake controls differed from the corresponding experimental vessels only in the absence of substrate. The *net oxygen uptake curves* were obtained by subtracting the mean endogenous oxygen consumption of the controls from the mean oxygen consumption of the experimental vessels for each reading. All flasks, experimentals, controls and thermobarometers, were run in duplicate. When substrate autoxidation controls were run, 1 ml of 0.9% NaCl was substituted for skin homogenate in the main portion of the reaction vessel.

Control homogenates in the absence of iodoacetamide showed a marked tendency to increase their oxygen consumption rates abruptly after several hours of incubation, sometimes even surpassing the oxygen consumption in experimental vessels. For this reason it was decided to rely primarily on net oxygen uptake computations obtained from experiments run in the presence of iodoacetamide, where oxygen consumption irregularities were very infrequently encountered.

Measures of tyrosinase and dopa oxidase activities were obtained by estimating maximum rates of net oxygen uptake. In the case of tyrosine, where an induction period was evident, the maximum rate was taken from the straight line portion of the S-shaped curve. The induction period was determined by extending the straight line portion until it intersected the time axis. The pooled results of our experiments reveal a significant negative correlation ($r = -0.65$) between maximum rate and induction period. In the case of dopa, with no induction period, maximum net oxygen uptake rates were obtained from the first two sets of readings, generally within the first $\frac{1}{2}$ hour of each experiment. In the case of certain very weakly active skins, such as "Red" or some agouti skins, oxygen consumption measurements could not be obtained, although a slow darkening over controls was noted on overnight incubation. A rate of 1 microliter/hr was arbitrarily assigned to these skins to indicate the existence of a low level of activity.

RESULTS

Demonstration of tyrosinase activity

Manometric and histochemical demonstrations of tyrosinase activity in hair bulbs of skin fragments have already been reported (FOSTER 1952a). Other criteria for

demonstrating the enzyme included specificity of the skin catalyst for the L-configuration of tyrosine. Furthermore, the catalyst was poisoned by the addition of phenylthiourea. Finally, both pigment production and oxygen consumption (especially the shortening of induction periods) were enhanced by iodoacetamide, presumably by destruction of inhibiting sulfhydryl compounds. A typical set of net oxygen uptake curves is shown in figure 1. In this case the skin of a 52 day old pale brown foetus had been used.

Comparison of activities of various genotypes

The results of individual experiments, involving the maximum net oxygen uptake of self-colored and agouti skins with tyrosine in the presence of iodoacetamide are shown in the form of frequency distributions in figure 2. These results, together with those obtained with dopa, as well as the results with tyrosine-negative skins, are summarized in table 1. Analysis of the results in table 1 reveals the following:

1. Substitution of *ff* for *F*

Although *ff* intense brown and black skins appear to show somewhat higher activity than corresponding *F* skins, the differences are not significant. These results

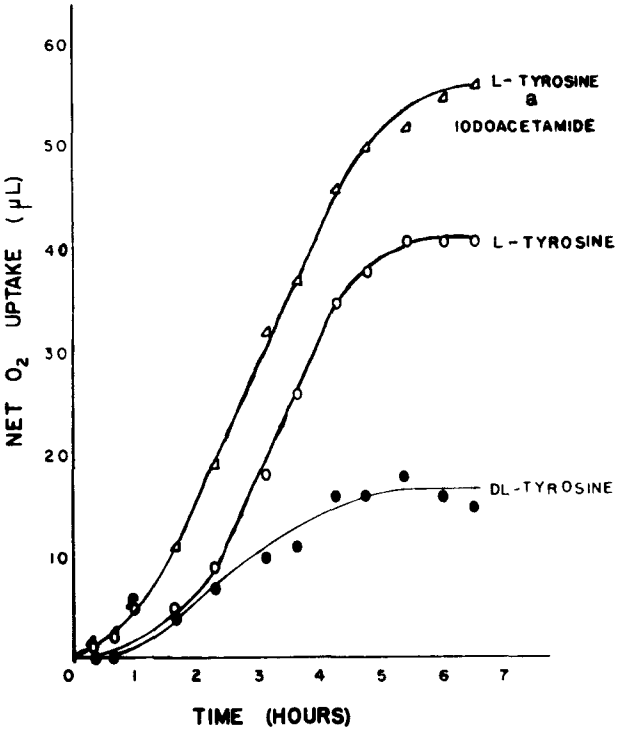

FIGURE 1.—Net oxygen consumption curves for a pale brown skin (*bbFfpp*) from a 52-day foetus, showing specificity for the L-configuration of tyrosine and enhancement of activity by iodoacetamide, especially in shortened induction period.

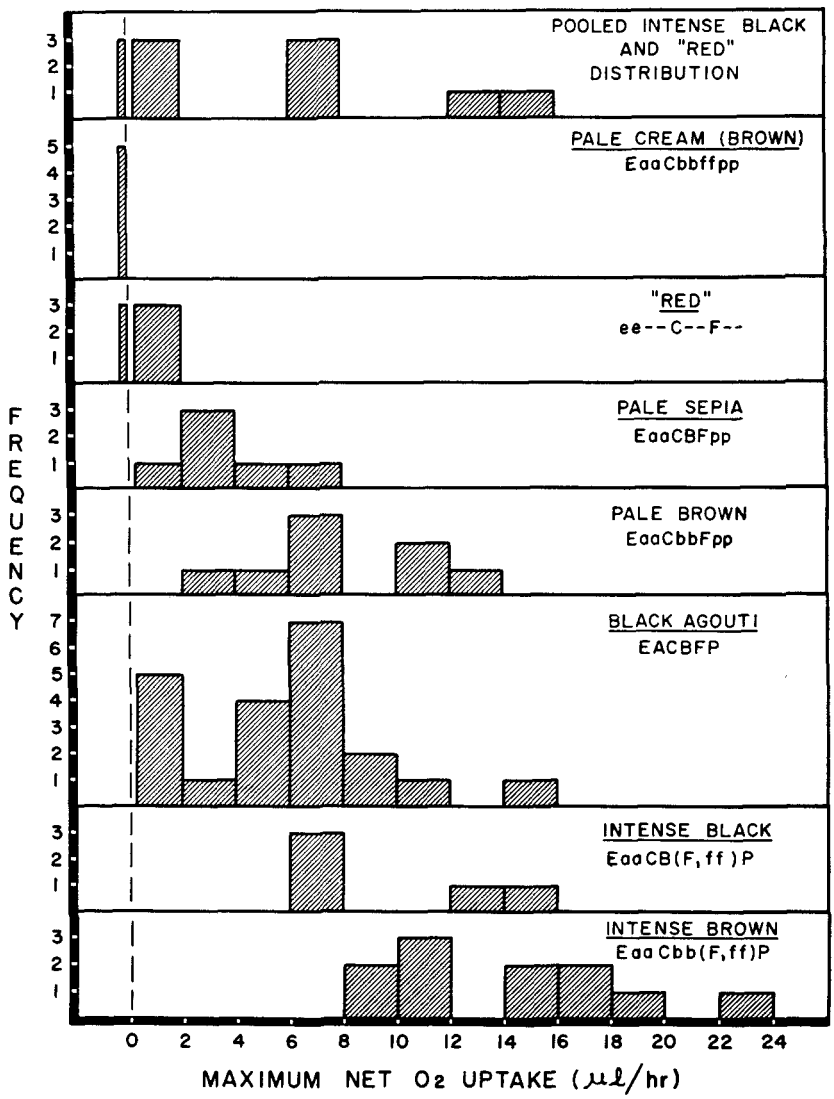

FIGURE 2.—Frequency distributions of tyrosinase activity measurements (maximum rate of net oxygen uptake in microliters/hr) of tyrosine- and/or dopa-positive skins of different genotypes.

were therefore pooled in order to permit other comparisons, and the pooled data are enclosed in the space bounded by double lines.

2. Substitution of *bb* for *B*

The average activity of the pooled intense browns towards tyrosine is greater than that of the pooled intense blacks, but not significantly so (the value for t gives a P value near the 10% level). The average activity of the pale browns toward tyrosine, however, is significantly higher than that of pale sepias. From these two sets of

TABLE 1

Tabulated summary of tyrosinase activity in various genotypes, as measured by maximum rate of net oxygen consumption in the presence of iodoacetamide

Genotype	Phenotype	Tyrosine			Dopa (.5 mg/ml)			Dopa (1 mg/ml)		
		M	SE	(N)	M	SE	(N)	M	SE	(N)
EaaCbbffP	Intense brown	15.9 ±	2.5	(5)	39.7 ±	1.6	(5)	68.5 ±	4.6	(5)
——B——	Intense black	13.6		(1)	40.5		(1)	58.5		(1)
——bbF—	Intense brown	12.8 ±	1.2	(6)	32.7 ±	10.2	(2)	45.2 ±	12.7	(2)
——B——	Intense black	9.2 ±	2.2	(4)	—			—		
EaaCbb (F,ff)P	Intense brown	14.2 ±	1.3	(11)	37.7 ±	2.8	(7)	61.8 ±	6.0	(7)
——B———	Intense black	10.1 ±	1.9	(5)	40.5		(1)	58.5		(1)
——bbFpp	Pale brown	8.3 ±	1.2	(8)	23.4 ±	2.6	(4)	31.0 ±	6.4	(3)
——B——	Pale sepia	3.7 ±	0.9	(6)	18.6 ±	1.4	(4)	27.2 ±	2.6	(4)
ee–C—F—	"Red"	0.5		(6)	7.1 ±	1.0	(3)	9.5 ±	0.9	(4)
EaaCbbffpp	Pale cream brown	0.0		(5)	5.9 ±	1.8	(5)	9.6 ±	2.4	(6)
EACBFP	Black agouti	5.7 ±	0.8	(21)	—			—		
c^kc^r, c^rc^r, c^kc^kpp, c^rc^a, c^dc^a, c^ac^a.	Black to white	0		(7)	0		(2)	0		(1)

comparisons it seems reasonable to conclude that a mutation from the black to the brown pigmented condition results in somewhat more demonstrable tyrosinase activity. This change runs counter to the change in amount of pigment, since brown hair contains only about 40% of the amount found in black hairs (WRIGHT 1949).

3. Substitution of *pp* for *P*

The average activity of intense browns towards tyrosine is significantly higher than that of pale browns, and the same holds true for a comparison of intense vs. pink-eyed blacks (pale sepias). Thus mutation from intense dark coloration to the pale, pink-eyed, condition involves parallel reductions in both enzyme activity and amount of eumelanin.

4. Substitution of lower albino series alleles for *C*

In our experience with the manometric technique the absence of gene *C* results in the complete loss of activity towards exogenous tyrosine or dopa, regardless of the amount of natural dark pigment present in the skin. For example, c^kc^r intense blacks are nearly phenotypically indistinguishable from the corresponding tyrosine-positive C- blacks, yet, paradoxically, their behavior in Warburg reaction vessels appears to be categorically different.

Two apparently exceptional cases were noted in relation to the lower albino series group. One foetus of a litter involving possible segregation of *ppC* pale sepias and

ppc^r*c*^{r} very pale sepias was classified as a very pale sepia. The skin of this foetus, however, in contrast to the tyrosine-negative results with all other skins of this group, gave a weakly positive reaction. PROFESSOR WRIGHT has kindly advised me that this foetus could well have been an abnormally light pale sepia, *ppC-* with additional incompletely defined dilution factors. Thus this case does not appear to contradict our other findings in the lower albino series group. We have not, however, included this case among the usual pale sepias, inasmuch as it would be an exceptionally weak variant in that category.

The other apparently exceptional case appears to have involved a misclassification. A dark foetus, originally classified as intense black (i.e., *C-B-*) gave no reaction towards tyrosine, in contrast to the marked activity of every other intense *C-B-* black. A histological comparison of a whole mount skin sample from this foetus with samples obtained from *C* and *c*^{k}*c*^{r} intense blacks indicated that the exceptional foetal skin was lighter than these intense blacks. PROFESSOR WRIGHT, on further examination of his breeding records, has informed me that segregation for sepia of genotype *c*^{r}*c*^{a} could have occurred in this litter. Thus a reasonable explanation is available for each of the apparently exceptional cases encountered.

5. Substitution of *ee* for *E*

"Red," or the most intense yellow, skins gave nearly negative results with tyrosine. Only a slow overnight darkening, and only in the presence of iodoacetamide, was evident in 3 out of 6 skins. A correspondingly low activity towards dopa was also observed. Thus the inherited color change from intense black or brown to "red" is accompanied by a drastic reduction in tyrosinase activity.

In order to determine whether the low demonstrable activity of red skin might be due to the presence of large amounts of sulfhydryl inhibitors, the effects of increasing the concentration of iodoacetamide were tested. No aditional enhancement was obtained, thus suggesting only low level of enzyme activity in red skin.

6. Substitution of *A* for *aa*

Of the 21 cases in the agouti group 15 were classified as agoutis on the basis of a histological examination of their skin samples, which showed a mixed population of black and red hair bulbs. The remaining 6 foetuses, obtained from a black self (*aa*) × agouti (*Aa*)mating, had been tested before the procedure of preparing whole mount skin samples had been adopted. The mean activity and the range for these 6 skins however, were nearly the same as for the other 15 identified skins. (Mean for the 6 unidentified skins: 5.5 microliter/hr, 5.7 microliter/hr for the 15 identified ones. Range for the 6 unidentified skins was 1.0–14.0 microliter/hr, 1.0–10.8 microliter/hr for the 15 identified ones.) It thus appears justifiable to pool these results.

The average activity of agouti skins towards tyrosine was significantly lower than that of self-colored *C* blacks. Examination of the frequency distribution of agouti skin activity (fig. 2) indicates two additional striking features—an extremely wide range and a bimodal distribution. Such a range and bimodality are approached by pooling the results obtained with the self-colored *C* blacks and "reds," as shown at the top of figure 2. Thus it appears that the net reaction of an agouti skin tends to fall into

one of the two major categories—an activity level characteristic of self-colored black, or a level characteristic of solid "red."

7. Factor interaction in *ffpp* (pale cream) genotypes

Of the 5 pale cream skins tested none showed activity towards tyrosine, and only the very weak activity towards dopa (significantly higher than the autoxidation level of about 2 microliter/hr) suggested the presence of a small amount of enzyme. As far as our data go, it appears that pale cream skins are even less active than "red" skins.

Especially striking is the phenomenon of factor interaction between genes *ff* and *pp*, affecting the level of demonstrable enzyme activity. Both *ff* intense brown and *pp* pale brown skins exhibit, respectively, very high and high levels of activity. In the double recessive pale cream (*ffppbb*) combination, however, enzyme activity has practically vanished. That is to say, enzyme activity in either of the two mutant types is high, but is practically eliminated when the two mutations in homozygous condition are combined in the same organism.

DISCUSSION

The dark-yellow problem

Our results, in relation to the dark-yellow problem fall into three categories:

1. Parallelism between tyrosinase activity and dark pigmentation

The parallel effects of gene substitution on tyrosinase activity and on the amount of natural dark pigment are evident in the cases of the *P* and *F* loci. Substitution of *pp* for *P* (in the presence of *F*) reduces both enzyme activity and amount of dark pigment, although it does not affect the amount of yellow pigment. Moreover, substitution of *ff* for *F* (in the presence of *P*), while reducing yellow pigment, reduces neither tyrosinase activity nor the amount of dark pigment.

The complementary relation between *ff* and *pp* in the nearly dopa-negative double recessive pale cream skin, with factor interaction affecting both eumelanin (but not phaeomelanin) and enzyme activity, suggests in addition the existence of some as yet unspecified metabolic connection between the synthesis of tyrosinase and the synthesis of yellow pigment. Clarification of this relationship must await more definite information concerning the enzymatic production of yellow pigment.

2. Reasonably explained cases

a. Although substitution of *bb* for *B* results in a marked decrease in amount of dark pigment, this genetic alteration certainly does not decrease and even seems to increase the activity towards exogenous tyrosine. If brown pigment is produced from tyrosine, reduced pigmentation can be explained by postulating some form of endogenous substrate limitation, with no effect on total tyrosinase activity, as was previously suggested by WRIGHT (1942). With a smaller portion of total enzyme combined with endogenous substrate in brown skin than in black, the greater activity of brown skin could then be due to the greater amount of enzyme available to act on exogenous substrate.

b. If dark and yellow pigments are produced from different precursors (granting

the existence of unspecified metabolic links between these two major pigmentary systems), the marked difference in tyrosinase activity between black and yellow skins needs no explanation. On the other hand, on the hypothesis that there is a parallel effect of gene substitution on the capacity to produce yellow pigment and on tyrosinase activity, the change from *E* to *ee* or from *aa* to *A* should involve no change in tyrosinase activity, since these gene changes involve no intensity modification of yellow pigmentary capacity. Yet our observations indicate striking changes in activity resulting from these gene substitutions. It is still possible, however, to assume with WRIGHT (1942) that tyrosine is a common precursor of both eumelanin and phaeomelanin and that significant divergence of the eu- and phaeo- melanic processes occurs at a stage in the reaction sequence subsequent to the oxidation of tyrosine. Experimental support of this view is furnished by the report that C-14 labeled tyrosine or some product of this is incorporated into phaeomelanic granules in the mouse (MARKERT 1955), although it has not yet been determined whether the labeled tyrosine is incorporated into the pigmented or non-pigmented portion of the yellow granules.

3. Unexplained

Clearly, the paradoxically tyrosine- and dopa-negative results obtained with the lower albino series group do not permit any rationalization of the lack of parallelism between tyrosinase activity and amount of dark pigment. In the absence of positive results with the manometric method two different possibilities may be entertained: 1) either tyrosinase is present in these skins, in which case the necessary conditions for its demonstration are drastically different from the conditions necessary for its demonstration in *C*-skins, or 2) in the absence of gene *C*, an enzyme system other than the tyrosinase system might produce dark pigment from tyrosine or some other chromogen.

Thus, although there are difficulties in considering the *C* locus, the observed effects of gene substitution at the other five major color loci together with the case of factor interaction between *ff* and *pp* either directly support, or can be reconciled with, the view that the tyrosinase system produces dark pigment, that yellow pigment might be produced from some chromogen other than tyrosine (although this question is very much open), and that these two major pigmentary systems are somehow metabolically linked.

Comparison with results of previous investigators

Although our conclusions are quite different from those advanced by W. L. RUSSELL and by GINSBURG, the various sets of observations show a good deal of agreement. Moreover, at least some of the differences in results can be attributed to differences in materials and methods.

Comparison with RUSSELL's results

The visual grading of the dopa reaction, while having the advantage of permitting observation of a localized reaction, suffers from three major shortcomings as contrasted with the manometric method: (1) It involves shifting standards for comparing activities of differently colored skins, i.e., black vs. yellow, pale sepia or pale

cream. (2) A linear scale of visual grades probably corresponds to exponential increases in amounts of pigment (WRIGHT and BRADDOCK 1949). (3) Dopa is much more readily transformed into melanin than is its more stable precursor, tyrosine. (On the other hand, the visual grading method is probably more sensitive in the detection of weak reactions on lightly pigmented backgrounds.) Thus this assay method probably imposes a lower ceiling on the observer than does the manometric method, and this lower ceiling value is more often reached when dopa rather than tyrosine is used as the exogenous melanogenic substrate.

As regards visual assays for hair bulb reactions, it is not surprising, then, that while there is a general correspondence with our results, there is a general relative upgrading of the less active skins. Thus yellow hair bulbs seem only slightly less active than black or brown, with no apparent reduction in pale (*pp*) genotypes, and various lower albino series allelic combinations exhibit only reduced activity as compared with the absence of demonstrable activity with the manometric method. It is therefore possible to reconcile all our major results except in the case of *ff* intense genotypes, where RUSSELL reported a sharp reduction in enzyme activity while no such reduction was observed manometrically.

As for the dopa reaction of the basal epidermal melanocytes the dark-yellow problem vanishes, since these cells appear to contain only eumelanin, even when the coat color is yellow (W. L. RUSSELL 1939). The correspondence between these and our results is almost perfect, such as greatly reduced or no reaction in the cases of lower albino series combinations, *pp* and *ffpp* genotypes, as well as the presence of full activity in *ff* dark genotypes. In our preparations of homogenized foetal skin, however, the vast majority of the melanocytes are to be found in the hair bulbs. (DR. WILLYS K. SILVERS has kindly shown me histological preparations of dorsal skin from a 55-day foetus, and the numerical preponderance of hair bulb melanocytes is obvious.) Thus the interesting possibility arises that the hair bulb melanocytes of foetuses might behave enzymatically more like those of the basal epidermal class than of the hair bulb class in animals at least 30 days after birth.

Comparison with GINSBURG's results

Our results, with two apparent exceptions, agree well with those reported by GINSBURG for his "immediate" extracts, i.e., no reaction in the absence of gene *C*, sharply reduced activity on substitution of *ee* for *E* and the lack of reduced activity on substitution from *B* to *bb*. The only important apparent differences are found in the cases of *ff*, where GINSBURG noted reduced activity, and of *pp*, where he reported no reduced activity—the reverse of our results. However, reductions due to single or double doses of gene *f* were most readily observed by GINSBURG in yellow (*ee*) genotypes, which we have not yet studied. Moreover, the apparent lack of reduced dopa oxidase activity in *pp* extracts might be due to a lower ceiling of measurable effect in the turbidimetric method.

Additional storage of GINSBURG's skin extracts in a refrigerator ("delayed" extracts) resulted, for all cases excepting unpigmented skin, in increased production of dopa melanin, thereby bringing them into greater correspondence with RUSSELL's results involving hair bulb reactions. Thus some of the lower albino series skin ex-

tracts, inactive as "immediate" extracts, became active as "delayed" extracts. (Similar results have recently been reported by HOROWITZ and FLING, 1954, in connection with extracts from Drosophila.) The similarities and differences between our results and these are about the same as obtain in the comparison between our results and RUSSELL's hair bulb assays. Here again some of the differences may in part be due to the use of dopa as exogenous substrate in one study and of tyrosine in the other.

The agouti pattern

The yellow-banded agouti color pattern has been the subject of various recent investigations, and it seems desirable to summarize and correlate the results of these studies. Substitution of gene *A* for *aa* results in a pigmentary pattern characterized by a subterminal phaeomelanic band in an otherwise dark hair. There is thus a strict correlation between the stage of the hair growth cycle and melanocyte function. The histological studies of E. S. RUSSELL (1949) in the mouse lead to the view of a reversible switch mechanism involved in agouti banding, a replacement of the eumelanic process by the phaeomelanic process (rather than a temporary unmasking of yellow pigment) followed by a resumption of the dark pigmentary process. The transplantation studies of SILVERS and RUSSELL (1955) indicate that the kind of pigment produced by hair bulb melanocytes is determined by the follicular environment, which in turn is conditioned by genes at the agouti locus in the mouse.

As regards the biochemical aspects, DANNEEL (1949), reviewing his and other studies on rabbits, viewed the yellow band as a possible consequence of a temporary inhibition of dopa oxidase activity or of a temporary drastic reduction in the rate of dopa oxidase synthesis. FOSTER (1951), comparing the oxygen consumption patterns of solid yellow, solid brown and brown agouti mouse skin homogenates incubated in tyrosine, suggested that yellow band formation might be due to the rather abrupt suspension and resumption of tyrosinase activity as a consequence of sharp fluctuations in the level of a tyrosinase inhibitor. Our present guinea pig data, however, do not lend themselves as readily to such a conclusion, because the corresponding guinea pig genotypes differ in their oxygen consumption patterns from those of the mouse in that, in all cases so far tested, guinea pig skin homogenates incubated in tyrosine exhibit an induction period. Suggestive evidence for the implication of sulfhydryl inhibitors has been reported by CLEFFMANN (1953) in the agouti rabbit. Moreover, CLEFFMANN (1954), growing mouse skin in tissue culture, reported both the induced production of yellow pigment in black self skin by the addition of glutathione to the medium and the partial suppression of yellow pigment formation in agouti skin by the addition of sulfhydryl-destroying iodoacetamide to the medium.

Correlations between yellow band formation and stage of hair growth cycle are provided by DANNEEL (1949) and CLEFFMANN (1953), who reported that in the rabbit the yellow band occurs in a thickened region of the hair. Moreover, CLEFFMANN (1953) noted that this period of increased growth rate in agouti rabbit hair is accompanied by an increased mitotic index in the cells of the hair matrix, although no comparable control data are given for corresponding developmental stages of solid black hairs. Finally, a characteristic feature of the keratinization process in hair

growth is the oxidation of —SH groups to form cross linking disulfide groups (see GIROUD and LEBLOND 1951).

An attempted synthesis of the above observations leads to the tentative view that the gene controlled period of yellow band formation is marked by some rhythmic deviation in the keratinization process, with greater production of —SH compounds in the melanocyte environment than occurs in the case of non-agouti hair. The temporary increase in —SH groups inhibits tyrosinase activity and, perhaps, tyrosinase synthesis as well, thereby temporarily diverting pigment formation in the direction of phaeomelanin production. The introduction of —SH inhibitors into the melanocytes might be part of a reciprocal exchange between melanocytes and the developing hair cells migrating past them, the melanocytes "inoculating" the hair cells with pigment (see CHASE, RAUCH and SMITH 1951) and the —SH compounds passing from hair cells into melanocytes, perhaps via the same dendritic processes. The difficulty in visualizing the gene-controlled mechanisms involved in the onset and termination of this rhythmic pattern producing fluctuation still remains, however.

CONCLUSIONS

The major obstacle to the formulation of a scheme of gene substitution effects is our ignorance concerning the nature of the phaeomelanic process. A second obstacle is the apparently paradoxical behavior of lower albino series combinations. Whether it be assumed that the eumelanic and phaeomelanic processes diverge from a point of common substrate or of common enzyme precursor, it still appears necessary to attribute additional properties to component parts of these processes (such as thresholds, competition, inhibition, differences in reaction rates, formation of active or inactive complexes, or alternate pathways) in order to harmonize assumed changes in single-step reactions with observed phenotypic effects. Thus on the assumption of common enzyme precursor one complication arises in considering the effects of *ff* and *pp*, acting separately on the synthesis of a yellow producing enzyme or of tyrosinase, respectively, and also interacting to affect the eumelanic system. On the assumption of common melanogenic substrate, the dual effects of albino allelic substitutions on both eumelanic and phaeomelanic processes introduce a complication. In any event, in this study involving attempts to insert a metabolic phenotype between genotype and phenotype observed at higher organizational levels, it appears that the various gene substitutions affect various components of a ramifying metabolic network. Thus some mutations appear to affect primarily the rate of tyrosinase synthesis (i.e., *e* and *p*), another mutation might somehow affect the amount of available tyrosine (*b*), another the periodic excess of a tyrosinase inhibitor (*A*) in the melanocyte environment, still another might differentially affect both tyrosinase synthesis and phaeomelanin production (*C* series), yet another might affect primarily the phaeomelanic process (*f*), and finally two different biochemical lesions (*ffpp*) somehow additionally affect tyrosinase synthesis.

SUMMARY

1. Inherited variations in the tyrosinase system in foetal guinea pig skin, in which hair bulb melanocytes greatly outnumber basal epidermal melanocytes, have been

studied by means of oxygen consumption measurements of individual skin homogenates incubated in tyrosine and also, less frequently, in dopa.

2. The observed effects of gene substitution are as follows: (a) Replacement of *P* by *pp*; which reduces the amount of dark pigment (eumelanin), but not of yellow pigment (phaeomelanin); results in decreased tyrosinase activity. (b) Replacement of *F* by *ff*; which reduces the amount of phaeomelanin, but not of eumelanin; results in no decreased tyrosinase activity. (c) Simultaneous replacement of *F-P-* by *ffpp*; involving factor interaction causing virtually complete elimination of eumelanin, but not of phaeomelanin; results in no demonstrable tyrosinase activity and barely demonstrable dopa oxidase activity. (d) Replacement of *B* by *bb*; which reduces the amount of eumelanin, but not of phaeomelanin; results in no decrease in tyrosinase activity. (e) Replacement of *E* by *ee*, involving the substitution of yellow for a dark coat, results in a drastic reduction in tyrosinase activity to a barely demonstrable level. (f) Replacement of *aa* by *A*, involving subterminal yellow bands, results in a very wide range and a bimodal distribution of tyrosinase activity measurements. The tendency of these measurements of black agouti skins to cluster about the values characteristic of solid yellow and of solid black skins suggests that the net activity measured for each agouti skin is a function of the relative number of hair bulb melanocytes engaged in yellow pigment formation at the time of enzyme assay. (g) Replacement of *C* by any combination of lower alleles of the series, with differential diluting effects on both eumelanin and phaeomelanin, results in the apparently complete absence of both tyrosinase and dopa oxidase activities. These paradoxically negative results, especially in such intensely black skins as $c^k c^r$, might in part be due to a high threshold for enzyme demonstration inherent in the manometric technique. Drastically reduced activity in all skins lacking gene *C* is, however, certain.

3. When allowances are made for differences in materials and methods, there is quite good agreement between the present results and those previously obtained by W. L. RUSSELL (1939) and by GINSBURG (1944).

4. The present results, however (except for the *C* series), can be considered as either directly supporting or at least reconcilable with the view that the manometric method measures inherited capacity to produce dark pigment. Directly supporting evidence is found in the cases of *ff*, *pp* and *ffpp* genotypes. The lack of reduced activity in brown skin, however, seems to require the assumption of endogenous substrate limitation resulting from substitution of *bb* for *B*. The very slight tyrosinase activity of yellow hair bulbs (in *ee* or *A* skins) may be interpreted in two ways: (a) yellow pigment is derived from a chromogen other than tyrosine or (b) yellow pigment is derived from tyrosine, but that significant divergence of the eu- and phaeomelanic processes occurs at some stage subsequent to tyrosine oxidation. Finally, the observed factor interaction on the chemical level in pale cream skin (*ffpp*) indicates the existence of some metabolic link between the synthesis of tyrosinase and the production of yellow pigment, thus supporting previously advanced conclusions based on genetic evidence (WRIGHT 1916).

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