INVESTIGATION OF THE PHYSIOLOGICAL GENETICS OF HAIR AND SKIN COLOR IN THE GUINEA PIG BY MEANS OF THE DOPA REACTION[1]

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INTRODUCTION

KNOWLEDGE of the processes by which gene replacements bring about character differences is still meager. Although the coat color of mammals was one of the first characters studied in the field of physiological genetics little has been discovered about the effects of gene substitutions on the pigmentation process. That these genic effects are suitable for further investigation, however, is suggested by the following facts which indicate that pigment formation in mammalian hair is comparatively simple from both a genetic and a physiological point of view. Color differences seem to be due largely to a few genes with major effects. Phenotypes are not greatly changed by environmental differences within the laboratory. Pigment appears when the hair follicles are first formed and is produced continuously throughout life. The persistence of mutant spots and the autonomy of transplants indicate that many genetic differences in color are determined locally. The character itself is simple, being merely an intracellular substance, melanin. The quantity and quality of this substance can be estimated visually. Since melanin is relatively stable it can be extracted. The amount of melanin in the extract can be determined gravimetrically, titrimetrically or colorimetrically. The essential feature in melanogenesis is believed to be an oxidation reaction controlled by enzymes.

Among mammals the guinea pig is particularly favorable for study because WRIGHT (1916, 1917, 1925, 1927) has presented a genetic analysis which is more extensive than that available for any other mammal. In itself this analysis has led to conclusions which impose definite limitations on any interpretation as to the processes involved in color production and the points in these processes at which the color genes have their primary effects. Through the work of RUSSELL (1939) and HEIDENTHAL (in press), who obtained measurements of the amounts of melanin produced by most of the color combinations studied by WRIGHT, the restrictions on theory

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have been made more quantitative than was possible from visual grading of hair color. The next step is to relate this information to a direct biochemical study of the various color combinations. This paper is presented as a contribution to such a study.

The term "melanin" is used to describe what is probably a group of related substances with similar properties. The chemical constitution of these is not known, but the nature of their formation in plants and animals has been demonstrated. At the beginning of the century it was shown that certain plants and animals possess oxidizing enzymes capable of producing melanins from melanogens (colorless organic cyclic complexes such as tyrosine). Since then similar results have been obtained from many plant and animal species and several authors have written extensive reviews on the subject. The general conditions necessary for the formation of melanin have been reviewed by SCHMALFUSS and WERNER (1926) and by MULZER and SCHMALFUSS (1933). It is generally accepted that the production of melanin in mammals resembles that in other forms, at least to the extent of involving an oxidizing enzyme and a melanogen.

By treating the skin of mammals with a standard concentration of a suitable melanogen solution, under controlled conditions, various workers have shown that differences between certain color varieties may be due simply to differences in enzyme content or activity. KRÖNING (1930) has used this method on guinea pigs. Since any theory as to the action of color genes is bound to consider facts of this nature it seemed desirable to make a more extensive investigation in this direction, with particular reference to the gene combinations studied by WRIGHT.

METHODS

Choice of method

In investigating the role played by enzymes in pigmentation in mammals several melanogens and ways of treating the skin have been employed. It was decided to choose from among these one relatively simple, sensitive and reliable method and to concentrate on this, using as many different genotypes as possible, rather than to examine a few genotypes with a multiplicity of biochemical tests. The results of earlier workers and the author's own findings regarding the merits of various methods are presented in this section.

Melanogens. Of the melanogens available only those two which have been used extensively, tyrosine and dopa (l. 3,4-dioxyphenylalanine), will be discussed here. For a fuller treatment see BLOCH (1927) and MULZER and SCHMALFUSS (1933).

Numerous workers have tried to obtain a reaction to tyrosine by pigment-forming cells in frozen sections, but the results have always been

negative. On the other hand, some authors claim that extracts from certain pigmented types will darken tyrosine while extracts from unpigmented animals will not. It is to be noted, however, that even this method has proved unreliable and that, in general, reactions were obtained only with difficulty, after prolonged time or by the addition of other substances. ONSLOW (1915) was apparently satisfied with the results obtained by the addition of hydrogen peroxide to extracts of rabbit and mouse skins tested with tyrosine. PUGH (1933), working with black rabbits, claims darkening of tyrosine, after 36 hours, in only two out of a large series of experiments and questions the validity of the conclusions which ONSLOW reached from his experiments. CHARLES (1938) also criticizes ONSLOW'S work and reports a tyrosine reaction in two out of three experiments with black mice.

The claims of earlier workers, that frozen sections will not react to tyrosine, were confirmed, at least for conditions under which the same tissue will react to dopa. In the following experiment both tyrosine alone and tyrosine plus dopa (the addition of dopa, as a possible priming agent for the tyrosine reaction, was suggested to me by DR. CHARLES) were tested against the appropriate controls:

Tyrosine stock solution: 0.400 gms in 1000 cc.
Dopa stock solution: 1.000 gms in 1000 cc.
The following solutions were made up:
A. 25 cc tyrosine stock plus 0.5 cc dopa stock.
B. 25 cc tyrosine stock plus 0.5 cc distilled water.
C. 25 cc distilled water plus 0.5 cc dopa stock.
Frozen sections from a black-eyed red guinea pig were tested in the following mixtures at pH 7.4 and at 38° C:
1. 10 cc A plus 10 cc buffer.
2. 10 cc B plus 10 cc buffer.
3. 10 cc C plus 10 cc buffer.
4. 10 cc dopa stock plus 10 cc buffer.
5. 10 cc distilled water plus 10 cc buffer.
Sections were removed and examined after three hours and after six hours.

The following results were obtained. Tyrosine alone (2) and its control (5) showed no reaction; tyrosine plus dopa (1) showed a very weak reaction which was, however, the same as that obtained with the same concentration of dopa alone (3); dopa alone in higher concentration (4) showed a strong reaction. The experiment was repeated and gave the same result.

Tyrosine was also tested in the presence of hydrogen peroxide in a concentration of one part of peroxide in 200 parts of the buffered mixture. Frozen sections gave no reaction in this mixture while other sections from the same piece of tissue gave a strong reaction with dopa.

It is, of course, possible that a reaction of frozen sections to tyrosine would occur under other conditions, but for the present purpose it is

sufficient to know that pigment-forming cells act more readily on dopa than on tyrosine.

A review of the conditions under which the melanoblasts of mammalian skin will react to dopa is given by BLOCH (1927). According to BLOCH the reaction occurs only when pigment is being formed and the degree of reaction is proportional to the pigment-forming activity. On the basis of this and other evidence BLOCH suggested that cells which have the capacity to produce melanin possess an oxidase ("dopa-oxidase") which is specific to dopa. BLOCH'S results have been substantiated by many workers and it is generally accepted that the reaction is enzymic, but the specificity attributed by BLOCH to dopa-oxidase has met with severe criticism, a short summary of which is given by FRANKE (1934). This criticism is based on the contention that the ease with which the reaction occurs may depend on the ease with which dopa is known to oxidize rather than on the specificity of the enzyme present. At the present time there is not sufficient evidence to determine whether the dopa and tyrosine reactions of mammalian melanoblasts are due to the same enzyme, to different enzymes or even partly to the same enzyme and partly to different enzymes. The demonstration by RAPER (1928, 1932) that dopa is an intermediate product in the artificial formation of melanin from tyrosine is, however, of interest in this connection. The fact remains that dopa, whatever its role, if any, in normal pigmentation in mammals, serves as a sensitive indicator of some phase of melanin-forming activity or capacity. It has been used by many investigators on diverse problems for over two decades and no other melanogen tested on mammals has given a stronger reaction. MULZER and SCHMALFUSS (1931) claimed that oxytyramine gave as marked a reaction as dopa in frozen sections of the ear of a black guinea pig. Since they used borax in their buffer mixture, and since in the present work it is shown that borax has a marked inhibiting effect on the dopa reaction, their conclusion cannot be accepted until the work has been repeated with a suitable buffer.

On the basis of the above findings dopa was selected as the melanogen for this investigation.

Treating the skin. In treating the skin with melanogen two methods, already mentioned above, have been used extensively: (a) An extract is made either by grinding the skin or by freezing it and then breaking it up with pestle and mortar. With the addition of water, followed by pressing, centrifuging, filtering or similar treatment, a more or less clear liquid is obtained to which the melanogen may be added. (b) Fresh or fixed skin is sectioned on the freezing microtome (the procedures of paraffin and celloidin embedding destroy the desired reaction) and the sections immersed in a solution of the chromogen.

Two other methods have been described. BECKER, PRAVER and THATCHER (1935) found that, given sufficient time, a solution of dopa will penetrate fresh tissue. Since sectioning follows the reaction paraffin embedding can be used. The other method employs a compress. As described by SCHULMANN and KITCHEVATZ (1926) this consists of several layers of filter paper saturated in dopa solution and enclosed within a small oiled-silk envelope which has an opening cut in one side. This is applied for from five to ten hours to the living animal on an area of the skin from which the superficial layers of the epidermis have been removed by scraping.

It seems to the author that use of the extract method has the following disadvantages. Tyrosine has given unreliable results and if dopa is used there is the difficulty that dopa reacts not only with the melanoblasts, but also, as cited by BLOCH (1927), with polymorphonuclear leucocytes, both in pigmented and unpigmented skin. Presumably it was this difficulty which was encountered by CHARLES (1938) and other workers who failed to obtain a clear-cut difference in dopa reaction of extracts from pigmented and unpigmented animals. Melanoblasts occur principally in the hair bulbs and in the basal layer of the epidermis. Even if an extract free from leucocytes could be obtained there is the further objection that, in a single extract, no distinction could be made between the reaction of the basal layer of the epidermis and that of the hair bulbs, reactions which, as reported in this paper, are often quite different.

The method worked out by BECKER and his collaborators, and described above, was designed to give thin sections for histological study of the basal layer of the epidermis. For comparing reactions in hair bulbs it was found to be not as suitable as the frozen section technique.

The compress method is of no use in measuring the reaction of hair bulbs. Even as a measure of the basal layer reaction it would seem to have two serious objections: first, the difficulty of scraping deep enough to obtain a reaction and yet avoid bleeding, and second, the difficulty of standardizing the scraping to give good comparisons.

The frozen section method was, therefore, chosen for this work.

Details of method

With the choice of the dopa reaction in frozen sections as the method of attack, an attempt was made to find the most favorable conditions for demonstrating any differences in reaction between the different genotypes. Since WRIGHT'S work has been concerned mostly with coat color the hair-bulb reaction was given primary attention. The techniques of other workers were compared and controversial points examined experimentally. The following factors were considered.

1. Age. STEINER-WOURLISCH (1925) claims to have shown that, in the mouse, pigment formation, and with it the dopa reaction, exhibit a rhythmic course during the growth of the animal. BOYD (1932) has compared adult sheep with lambs not more than three days old. She concludes that with age the production of dopa-oxidase becomes restricted to the fiber bulbs (which apparently react as strongly as ever) and can no longer be demonstrated in any great amount in the basal layer and follicle sheath. Preliminary experiments were conducted to discover whether or not the dopa reaction of hair bulbs in the guinea pig varies to any extent with age. With red animals examined at 15 different ages between a 44-day embryo and a 593-day adult there was very little difference in reaction. Since the coat color of some genotypes changes with increasing age, however, animals have, whenever possible, been tested before they were two months old.

Age in the growth cycle of individual hairs should not be confused with age of animal. Hairs in or near the club stage do not react with dopa. In a few cases a test had to be rejected because all hairs in the sample of skin sectioned were in this unfavorable stage of development. According to DAWSON (1930) the percentage of growing hairs in the guinea pig is greatest in March–April and October, and smallest in December and August, but the generality of this conclusion is questionable, for the author did not, as far as can be judged, use more than a few animals and the ages of these were not recorded. Examination of material used in the present work showed no simple seasonal influence.

2. Plucking. SCHULTZ (1925) and KRÖNING (1930) plucked their animals 10–14 days before the skin was tested, in order, so they claim, to obtain follicles in the same stage of growth in all experiments. The present author's observation was that plucking did not produce this result. Furthermore, in obtaining a set of data that can be compared with WRIGHT'S grades plucking is undesirable because certain genotypes change color after plucking. Plucking was, therefore, avoided.

Since hairs are not uniform in color along their length it is probable that the dopa reaction varies to some extent with stage of development of follicles. Apart from avoiding hairs approaching the club stage this possible source of variation was controlled only by observing large numbers of follicles.

3. Fixation. Many workers have tried both fresh and formalin-fixed skin and have preferred the latter because it permits neater sections. The time of fixation recommended varies from one to five hours in five percent formalin. BLOCH states that material which has been in this fixative for three days can still be used. Experiments by the present author showed that after two days in five percent formalin the reaction of the hair bulbs

was apparently as strong as after two hours; after four days the reaction was weak; after eight days it had ceased to occur. In a comparison of fresh and fixed material no great difference in the rate of reaction could be discerned, but the fixed material seemed to show a reaction a little earlier than the unfixed. Two hours fixation in five percent formalin was adopted.

4. Embedding. BLOCH embedded in agar agar solution. Good sections can, however, be obtained without embedding and so this step has been avoided.

5. Thickness of sections. In comparing the hair-bulb reaction of different genotypes under the microscope it seemed best to use either the whole bulb or a thin section through the center. Sections of skin cut thick enough (200 microns and above) to show whole hair bulbs were found to be unsatisfactory. Thinner (30 micron) sections were, therefore, used and comparisons limited to those bulbs which showed at least a portion of the central papilla.

6. Purity of solutions. BLOCH purified the water used in his work by repeated distillation, and he maintained that all trace of carbon dioxide must be removed before the solutions are made up. PERCIVAL and STEWART (1930) recommend distilling successively with potassium permanganate, phosphoric acid, sodium carbonate, and finally alone. LAIDLAW and BLACKBERG (1932) claim that such precautions are unnecessary; they simply distil tap water once with potassium permanganate and take no steps against carbon dioxide. Distilling ordinary laboratory distilled water once with potassium permanganate has given consistent results in the work reported here. No precautions were taken against carbon dioxide since it was found that even the addition of sodium carbonate to sufficiently buffered dopa did not affect the reaction.

7. Buffers. As has been mentioned before, MULZER and SCHMALFUSS (1931) used a mixture containing borax. In experimenting with a wide range of hydrogen ion concentration it was found, in the present work, that a borax-phosphate mixture, when compared with the mixture of primary and secondary phosphates used by most authors, has a marked inhibiting effect on both the spontaneous oxidation of dopa and the reaction in frozen sections. This is in accord with the previously observed rule, quoted by MICHAELIS (1930), that borax reacts with substances containing two or more hydroxyl groups. In trying higher values of pH than can be obtained with phosphate mixtures a mixture of barbital and hydrochloric acid (MICHAELIS 1930) was used. Where their ranges overlapped, phosphate and barbital mixtures gave similar results.

Except in the preliminary experiments described above the phosphate mixture was used exclusively. The concentration of the mixture is given

in the summary of this section. Tests showed that this concentration was adequate to maintain the required pH.

8. Concentration of dopa. Concentrations of dopa varying from one part in 500 parts of the buffered mixture to one part in 2000 have been recommended by different authors. In preliminary experiments the weakest of these concentrations appeared adequate and was used for the rest of this work.

9. pH. BLOCH, and most workers, used a pH of from 7.3 to 7.4. Some authors, namely FRANKE, state that this is the optimum pH for the enzyme. This is probably a misunderstanding of BLOCH, who said that the reaction may proceed faster in more alkaline solution, but that a pH higher than 7.4 is undesirable because of the increased non-enzymic oxidation of dopa and the consequent deposition of the melanin so formed on the sections immersed in the solution. In experimenting on this point frozen sections were tested at pH values ranging from 5.6 to 9.8, using the phosphate and barbital buffers already mentioned. The results indicated an optimum just above pH 8. However, BLOCH's claim that a lower pH gives better differentiation between melanoblasts and their surroundings was substantiated. A pH of 7.4 has been used throughout.

10. Temperature. Most, if not all, workers have used either room temperature or blood temperature. The reaction proceeds faster at blood temperature and is, therefore, less likely to be affected by disturbing influences from the outside. MULZER and SCHMALFUSS (1933) state that pigmentation reactions in general have their optimum between 20°C and 60°C. 40°C was chosen for this work.

11. Changing the solution during the reaction. If more than a trace of formalin is carried over into the dopa solution it is liable to inhibit the reaction. This seems to be due to a reaction between formalin and dopa. In test-tube experiments on the spontaneous darkening of buffered dopa, in the concentration used in this work, it was found that if formalin is present in the mixture in a concentration of 0.05 percent the solution will turn orange instead of producing a dark melanin precipitate. Even a concentration of 0.005 percent is not without visible effect.

LAIDLAW and BLACKBERG (1932) recommend replacing the original solution with fresh during the reaction. This has the advantage of removing any formalin that may have come in and also prevents the accumulation of an excessive amount of the melanin formed spontaneously by the solution. After a few experiments it was decided to change the solution at half an hour and also at an hour and a half after the sections were first immersed.

12. Time. With the above conditions the reaction is first clearly visible after about an hour and a half, and the general darkening of the sections

begins to obscure the melanoblast reaction after about six hours. The comparisons in this work were made on sections treated for three hours.

Summary

Since the above treatment of method has involved considerable discussion the actual procedures adopted are gathered together in the following paragraph.

The animal was anaesthetized with ether and a small piece of skin removed from the mid-dorsal line in the lumbar region. This was fixed in five percent formalin for two hours, rinsed for a few seconds in distilled water and cut into 30 micron sections on the freezing microtome. The sections were cut at right angles to the surface of the skin and in the plane of the hair shafts. Forty to fifty sections were placed in a solution made up of 10 cc of a 1 in 1000 solution of dopa, plus 8 cc of a 2M/15 solution of secondary sodium phosphate, plus 2 cc of a 2M/15 solution of primary potassium phosphate. (This concentration of buffer gives a pH of 7.4.) Control sections were immersed in a similar mixture with 10 cc of distilled water in the place of the dopa solution. These mixtures were contained in crystallizing dishes 5 cm in diameter and 3 cm in depth. The water used for the solutions was obtained by redistilling ordinary laboratory distilled water once with potassium permanganate. The dopa solution was always made up less than 24 hours before use and mixed with the buffer only immediately before the sections were cut. Experimental and control solutions were kept at 40°C and the experimental solution was replaced with fresh at $\frac{1}{2}$ hour and $1\frac{1}{2}$ hours after the sections were first immersed. The replacing solutions were kept at 40°C and dopa and buffer mixed just before use. Sections were removed after three hours, rinsed in distilled water, passed through alcohols and xylol and mounted in balsam.

MATERIAL

The animals used came from an extensive series of experiments conducted by DR. SEWALL WRIGHT for the purpose of producing animals of as great a variety of known genotypes, with respect to color, as possible. The genotypes were determined by DR. WRIGHT on the basis of the genotype of the parents, the animal's own color and, in certain cases, breeding tests.

The colors of the animals used here are referred to usually only by genotype. In all tables each genotype is indicated by those genes in which it differs from the genetic formula $S-E-a\,a\,C-F-P-B-$ (symbols according to WRIGHT). For convenience in description the genotype represented by the above formula, in which is present the wild-type gene of every series of alleles except the $A-$ (agouti) series, will be referred to as "type."

Hair color

The colors of the various genotypes have been discussed extensively by WRIGHT (1927), but for convenience in comparing the dopa reaction with natural pigmentation table 5 has been prepared from his data. This table shows the average grades of coat color of the genotypes studied here and also of certain other genotypes. The inclusion of more than one genotype within a single column indicates that these genotypes have the same grades of coat color. The range of colors possible within each albino (*C* −, c^k c^k, etc.) series is given at the top of the columns. In grading, WRIGHT used two series of skins corresponding with the two apparently qualitatively different kinds of pigment which, in this paper, are denoted as sepia and yellow. Black, sepia, brown and pale sepia colors appear sufficiently alike in quality to be graded against a single series of standard skins ranging from grade 1 to grade 21. The remaining colors, red, yellow and cream, were graded against a series ranging from 1 to 13. In table 5, *e e*, *e e p p* and *e e b b* combinations are all homozygous for *F*. WRIGHT'S grades for *e e F f*, etc., combinations (not shown in table 5) were slightly less than for the corresponding *e e F F*, etc., combinations. Since the difference is so small animals used in the present work were not tested to determine whether they were *F F* or *F f*.

Red, yellow, cream, and similar animals showing "sootiness" (which usually develops with age) were avoided in the dopa work.

RESULTS

The method chosen has produced remarkably uniform results within each genotype. For example, including preliminary experiments 23 black-eyed red animals have been examined and of these five have been examined twice. Of these 28 tests not one failed to give a marked reaction. In a total of 13 white animals none gave a reaction. There is evidence which indicates that the variation remaining within each genotype is due more to variation in color of animals and stage of development of follicles than to the method.

Grading

Grading of both hair-bulb and basal-layer reactions was made as objective as possible by preventing the genotype of each animal from being known while the sections were being graded.

Hair bulbs. After comparison of experimental and control sections the material was divided into two groups.

The first group contains genotypes which showed so little pigment in hair bulbs of the control sections that it could not have contributed, measurably, to the grade of pigment shown in the experimental. This

TABLE 1

Distributions and means of the grades of dopa reaction in the hair bulbs.

	p p							*e e*							*e e e p p*						
	0	1	2	3	4	5	MEAN	0	1	2	3	4	5	MEAN	0	1	2	3	4	5	MEAN
						48	5.00					5	41	4.89				1	16		3.94
						24	5.00				73	3		3.04					4		4.00
C —						13	5.00					3		4.00			2	78			2.98
						81	5.00				1	11	4	4.19				4	89		3.96
												21	21	4.50					58	7	4.11
												52	8	4.13							
			8	23	1		2.78		2	12	3			2.06		15	22				1.59
$c^k c^k$				1	14	4	4.16		116					1.00		16	18	1			1.57
			17	30	4		2.75		2	1				1.33		3	35	5			2.05
			1	25	4		3.10		4	8	2			1.86							
$c^k c^d$			7	2			2.22									18	11				1.38
			6	2			2.25		32	11				1.26	2	32					0.94
$c^k c^r$		5	3				1.38		59					1.00							
		3	50	1			1.96														
		4	23	1			1.89														
$c^k c^a$			75	16			2.18								3	6					0.67
		10	29				1.74														
			39	83	5		2.73		3	20	8			2.16		2	5	4	1		2.33
$c^d c^d$									4	41	3			1.98			40	14			2.26
										2	16	4		3.09							
										19	5			2.21							
			3	4			2.57		10	8				1.44		28	5				1.15
$c^d c^r$		28	13				1.32														
		30	6				1.17														
		11	54				1.83														
$c^d c^a$			18	1			1.05			13	2			1.13			29	5	4		1.34
			13				1.00														
	51						0.00	9						0.00	27						0.00
$c^r c^r$	16						0.00														
	25						0.00														
	3						0.00	53						0.00							
$c^r c^a$	8						0.00														
	4						0.00														

TABLE 1 (*Continued*)

	f f p p							*e e f f*							*e e f f p p*						
	0	1	2	3	4	5	MEAN	0	1	2	3	4	5	MEAN	0	1	2	3	4	5	MEAN
			8	8			2.50		3	8	12			2.39			2	2			2.50
C —		1	13	6			2.25										2	7			2.78
																	3	30	56		3.60
$c^d c^d$								2	22					0.92	6	8					0.57
								13	23					0.64							

TABLE 1 (*Continued*)

	p p b b							*e e b b*						
	0	1	2	3	4	5	MEAN	0	1	2	3	4	5	MEAN
C –					17	14	4·45							
$c^k\ c^k$										2				2.00
								14						1.00

group is comprised of animals with red, yellow or cream natural pigment and the pale sepia (*p p*) and pale brown (*p p b b*) combinations. In the experimental sections of the above group every suitable bulb was graded under the microscope (magnification 100×) by comparison with a series of standard bulbs. The standards chosen represented six grades ranging from unpigmented (grade 0) to dark sepia or black (grade 5). As earlier workers have observed, the product of the reaction is always sepia in quality, even in red animals, and one series of standards was sufficient. Table 1 contains only the genotypes, mentioned above, in which the amount of natural pigment was negligible and in which the dopa reaction could, therefore, be graded directly. Within each genotype box in table 1 each row represents the reaction in a single animal. The figures show both the number of hair bulbs falling within each grade and, calculated from these, the mean reaction for each animal. Figure 1A shows a control section from that genotype in table 1 which has most natural pigment. That this amount of pigment may be disregarded when grading the amount of dopa-melanin formed is shown by figure 1B, an experimental section from the same animal. Two genotypes, $c^r\ c^r\ p\ p$ and $c^r\ c^a\ p\ p$, in table 1, recorded as giving no reaction, showed pigment in the experimental sections, but, as far as could be judged, no more than was present in the control sections. In these two cases it is, of course, possible that a very weak reaction would not be detected.

Results obtained from the second group of material, in which the amount of natural pigment was not negligible, are shown in table 2. Since the grades given were made by mentally subtracting the amount of pigment in the control from that in the experimental they cannot be regarded as more than approximate estimates of the actual reactions. Even when the control is extremely dark it is still possible to get an estimate from the fact that when a strong reaction occurs the bulb presents a solid black appearance, while the pigment in the control appears arranged in discrete granules or clumps of granules. An albino and the white area of a tri-color animal are listed in table 2 for convenience of tabulation although the control sections of these contained no pigment and, therefore, gave no difficulty in determining a lack of reaction.

Basal layer of the epidermis. Here the natural pigment appeared sepia

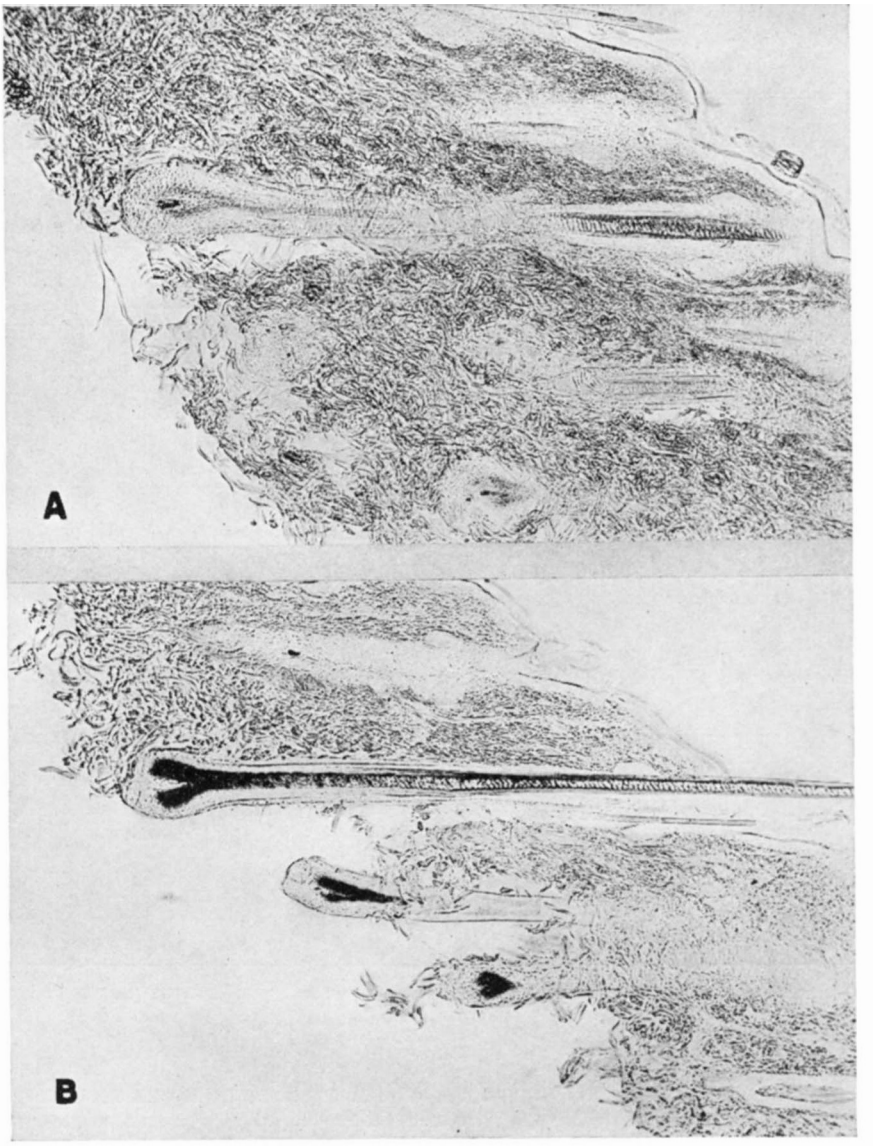

FIGURE 1.—A. Photomicrograph of a control section from an animal of genotype $p\ p$. (Magnification 60 diam.) 2. B. Photomicrograph of an experimental section, showing dopa reaction, from the same piece of skin that provided the control section shown in A. (Magnification 60 diam.)

or black even in animals with red hair color. Reactions were estimated by comparison with their controls. The results are given in table 3. Each row within each genotype box represents a single animal. There are two entries for each animal. The left entry indicates the amount of pigment in the control as judged by comparison with standards classified as: unpigmented

TABLE 2

Estimated dopa reaction in the hair bulbs of those genotypes in which accurate grading of the reaction is prevented by the presence of much natural pigment.

	TYPE	*f f*	*b b*	*A* —	*s s* e^p e^p	*s s* e^p e^p *b b*
C —	5 (2 animals: 39 and 165 hair bulbs)	2–4 (1 animal: 38 hair bulbs)		5* (1 animal: 144 hair bulbs)	Black area 5 (1 animal: 41 hair bulbs) / White area† o (1 animal: 19 hair bulbs)	Brown area 4–5 (1 animal: 155 hair bulbs)
c^d c^d	2–4 (1 animal: 40 hair bulbs)					
c^d c^r	2–4 (1 animal: 87 hair bulbs)					
c^r c^r	o–2 (1 animal: 13 hair bulbs)		o–2 (1 animal: 59 hair bulbs)			
c^a c^a	White area† o (1 animal: 20 hair bulbs)					

* Control showed 20 bulbs in the sepia-producing phase and 3 bulbs in the yellow-producing phase.

† Grading accurate in these cases.

(O), pale (P), medium (M) and dark (D). A trace of pigment occurring in two cases is indicated by O+. The right entry gives an estimate of the reaction graded as: no difference between experimental and control (O), a weak reaction (W), a strong reaction (S).

Skin color

Since extensive studies on the pigmentation of the epidermis in the different color varieties of the guinea pig are not available the following survey was made from the control sections listed in table 3.

It has been stated already that the pigment appears sepia or black, even in red animals. In most pigmented animals dendritic cells containing

pigment can be clearly seen in the basal layer and, in some animals, most, if not all, of the pigment present is confined to these cells.

TABLE 3

Natural pigmentation and dopa reaction in the epidermis.

	TYPE	*pp*	*ee*	*eepp*	*ff*	*ffpp*	*eeff*	*eeffpp*	*bb*	*ppbb*	*eebb*	*A* −	*sse*^{p}*e*^{p}	*sse*^{p}*e*^{p}*bb*
C −	D S M S	O O O O O W O O	P S P S P S P S M S M S M S	O O O+W O O O W O+W	M S	O O O O	P S	O O O O O O		O O		M S	Black area P S White area O O	Brown area P S
c^{k} *c*^{k}		O O O O O O O O	M O P O P O P O M O P O	O O O O O O							P O P O			
c^{k} *c*^{d}		O O		O O										
c^{k} *c*^{r}		O O O O O O O O	P O P O	O O										
c^{k} *c*^{a}		O O O O	P O	O O										
c^{d} *c*^{d}	D O	O O	P O P O P O P O P O	O O O O			P O P O	O O						
c^{d} *c*^{r}	P O	O O O O O O O O	P O	O O										
c^{d} *c*^{a}		O O O O	P O	O O				O O						
c^{r} *c*^{r}	P O	O O O O O O	P O	O O					P O					
c^{r} *c*^{a}		O O O O O O	P O											
c^{a} *c*^{a}	White area O O													

From table 3 it is apparent that the order of effect of the various genes on skin color is similar to that found in the eyes. WRIGHT'S determinations of the effects of the various genes on eye color can be described briefly as follows. There is no effect of *s s*, *e e* or *A* −. With respect to the albino

series, combinations involving c^k and c^d have little or no effect, but black is reduced to dark red by $c^r\,c^r$, to a light red by $c^r\,c^a$ and to pink (no pigment) by $c^a\,c^a$. It is important to note in the above that the order differs from that of sepia in the coat. There is no effect of $f\,f$. There is extremely little pigment left in the eye in $p\,p$ (eye pink). Finally, $b\,b$ reduces black to dark red or "brown." Combinations of two or more deviations from type have the effects expected. The actual pigment present in the eye in all cases is sepia in quality. Each of the following facts concerning skin color disagrees with the sepia, or the yellow, or both kinds of pigment in the hair, while it agrees with the pigment found in the eye: (a) the sepia quality of the pigment; (b) the absence, except for an occasional trace, of pigment in all combinations with $p\,p$; (c) the presence of pigment in $e\,e\,c^r\,c^r$ and $e\,e\,c^r\,c^a$; (d) the lack of reduction when $f\,f$ is substituted for $F-$. A possible contrast with the eye is in the effect of $E-$, but, with the amount of variation observed in the natural pigmentation of the epidermis, the evidence is not sufficient to determine whether the greater amount of pigment recorded in some $E-$ as compared with the corresponding $e\,e$ combinations indicates a real difference between these combinations or not. If the difference is real it is still possible that careful histological examination of the eye would reveal parallelism even here.

Dopa reaction in the hair bulbs

Table 4 was prepared from tables 1 and 2 to show the mean reaction for each genotype as calculated from the means for animals treated as units.

TABLE 4

Mean dopa reaction of the hair bulbs in each genotype.

	TYPE	*pp*	*bb*	*ppbb*	*ff*	*ffpp*	*A*−	*sse*^{p}*e*^{p} BLACK AREA	*sse*^{p}*e*^{p} WHITE AREA	*ee*	*eepp*	*eebb*	*eeff*	*eeffpp*
C −	(5)	5.00	(4–5)*	4.45	(2–4)	2.38	(5)	(5)	0.00	4.13	3.80		2.39	2.96
$c^k\,c^k$		3.20								1.56	1.74	1.50		
$c^k\,c^d$		2.22									1.38			
$c^k\,c^r$		1.87								1.13	0.94			
$c^k\,c^a$		1.96									0.67			
$c^d\,c^d$	(2–4)	2.73								2.36	2.30		0.78	0.57
$c^d\,c^r$	(2–4)	1.72								1.44	1.15			
$c^d\,c^a$		1.03								1.13	1.34			
$c^r\,c^r$	(0–2)	(0)	(0–2)							0.00	0.00			
$c^r\,c^a$		(0)								0.00				
$c^a\,c^a$	0.00													

Bracketed figures are estimates of the reaction in those genotypes in which accurate grading of the reaction is prevented by the natural pigment present.

* This estimate was made from the brown area of an animal of genotype *s s e*^{p} *e*^{p} *b b*.

The broad features of the data will be considered first. An effect of *s* in the combination *s s* e^v e^v is shown by the absence of any reaction in the white area of an animal that gave a full reaction in its black area. The mixture of yellow and sepia hair bulbs in the control of the agouti (*A* −) animal tested show that the hair bulbs in the experimental animal were probably of both types also. As far as they go the results indicate no difference in reaction between the two phases of color production found in this genotype. Combinations with *e e* in general show slightly less reaction than the corresponding combinations with *E* −. The effect of the *C* − series of alleles is to give three general levels of reaction: strong with *C* −; no reaction with c^r c^r, c^r c^a and c^a c^a; and intermediate with the

TABLE 5

Average grades of coat color as determined by WRIGHT.

	TYPE	*p p*	*bb*	*p p b b*	*f f*	*f f p p*	*e e e e p p e e b b*	*e e f f e e f f p p*
		BLACK SEPIA WHITE	PALE SEPIA WHITE	BROWN WHITE	PALE BROWN WHITE	BLACK SEPIA WHITE	PALE CREAM WHITE	RED YELLOW CREAM WHITE	YELLOW PALE CREAM WHITE
C −		21.0	9.7	15.6	8.3	20.9	2.2	10.6	6.8
c^k c^k		20.1	9.1	14.6	7.2	19.0		7.1	1.2
c^k c^d		19.4	7.6	15.4	6.0			7.2	
c^k c^r		20.5	7.0	14.8	6.1			4.6	
c^k c^a		18.5	5.5	13.6	5.6	18.0		4.6	0.0
c^d c^d		16.9	4.9	14.2	5.4	16.8	0.0	7.0	1.6
c^d c^r		19.1	4.0	15.0	3.7	18.6		4.1	0.2
c^d c^a		14.0	2.6	12.5	3.2	13.9		4.2	0.1
c^r c^r		20.1	2.6	15.5	2.8		0.0	0.0	0.0
c^r c^a		15.5	0.7	13.1	1.1			0.0	0.0
c^a c^a		0.0	0.0	0.0	0.0	0.0		0.0	0.0

remaining homozygotes and heterozygotes. Combinations with *f f* show less reaction than the corresponding ones with *F* −. Substituting *p p* for *P* − seems to have little or no effect on the reaction. If there is any effect from substituting *b b* for *B* − it is only a slight reduction.

The effects of the genes may now be considered in more detail. Tests of significance of the difference between *P* − and *p p*, between *E* − and *e e*, and between *F* − and *f f* combinations were made using STUDENT'S method for paired comparisons. The genotypes which were paired differed only in the alleles under consideration. Thus, in testing the significance of the difference between *P* − and *p p* combinations, *e e C* − *P* − (4.13) was paired with *e e C* − *p p* (3.80), *e e* c^k c^k *P* − (1.56) with *e e* c^k c^k *p p* (1.74) and similarly for eight comparisons in all. This test did not give a significant difference (P = 0.89). Testing *E* − against *e e* in nine com-

parisons showed a significant difference (P = 0.03). Testing $F-$ against ff in five comparisons gave a significant difference (P about .004).

Turning to the $C-$ series it is of interest to determine how many significantly different levels in intensity of reaction can be distinguished between the strong reaction given by $C-$ and the absence of reaction found with $c^r\ c^r$, $c^r\ c^a$ and $c^a\ c^a$. It will be seen from table 4 that the $C-$ series has been tested extensively in three combinations with other genes, namely: $p\ p$, $e\ e$ and $e\ e\ p\ p$. With $p\ p$ the order of reduction in reaction in the intermediate combinations is: $c^k\ c^k > c^d\ c^d > c^k\ c^d > c^k\ c^a > c^k\ c^r > c^d\ c^r > c^d\ c^a$. With $e\ e$ the order is: $c^d\ c^d > c^k\ c^k > c^d\ c^r > c^k\ c^r = c^d\ c^a$. With $e\ e\ p\ p$ the order is: $c^d\ c^d > c^k\ c^k > c^k\ c^d > c^d\ c^a > c^d\ c^r > c^k\ c^r > c^k\ c^a$. If an attempt is made to divide these intermediate genotypes into two levels of reaction it will be seen that there is only one line of cleavage which is consistent for all of the three groups. This places $c^k\ c^k$, $c^k\ c^d$ and $c^d\ c^d$ at the higher level and $c^k\ c^r$, $c^k\ c^a$, $c^d\ c^r$ and $c^d\ c^a$ at the lower level. A possible interpretation of this particular cleavage will be discussed later. The means for animals in the combinations $e\ e$ and $e\ e\ p\ p$, which, as already noted, do not differ significantly, were combined and the difference between the two levels indicated above was tested by STUDENT'S method for unpaired comparisons, following the conservative procedure of calculating the standard deviation from the combined data instead of from the deviations from the separate group means. (Standard error of the difference $= \sigma_T \sqrt{1/n_1 + 1/n_2}$, where σ_T = standard deviation of combined population, n_1 = number in first group, n_2 = number in second group.) The difference between the two levels (represented by 14 and 8 animals respectively) proved significant (P about 0.006). Similar tests between each of the intermediate levels and the $C-$ level (represented by 11 animals) also proved significant ($P < 0.001$ in each case). Thus, altogether, four significantly different levels of reactions were obtained with combinations of albino alleles in the presence of $e\ e$ and $e\ e\ p\ p$. For convenience these levels will be designated as: $C-$, $c^{k \text{ or } d}\ c^{k \text{ or } d}$, $c^{k \text{ or } d}\ c^{r \text{ or } a}$ and $c^{r \text{ or } a}\ c^{r \text{ or } a}$.

Dopa reaction in the basal layer of the epidermis

In white areas of spotted animals the basal layer of the epidermis, like the hair bulbs, shows no dopa reaction. The effect of the $C-$ series agrees in direction, but the threshold is higher (above all combinations except $C-$) than that (between $c^r\ c^r$ and $c^{k \text{ or } d}\ c^{r \text{ or } a}$) in the hair bulbs. Substituting $p\ p$ for $P-$ results in complete, or almost complete, loss of the reaction, whereas in the hair bulbs the same substitution has little or no effect. Substituting $e\ e$ for $E-$, or $A-$ for $a\ a$, has no apparent effect. Combinations with ff, as far as can be judged, show as strong a reaction as the corresponding combinations with $F-$, a result which differs from

that found in the hair bulbs. Finally, substituting *b b* for *B*— gives no recognizable reduction in the dopa reaction.

It should be noted that the differences between the reactions in the basal layer and the reactions in the hair bulbs are not all in the same direction. The lower members of the *C*— series and *p* give more reduction in the basal layer than in the hair bulbs (*p* none at all in the latter), while *e* and *f* give reduction in the hair bulbs but none in the basal layer.

DISCUSSION

Results of other workers

It is of interest to compare the results obtained here with those reported in rabbits by SCHULTZ (1925) and in guinea pigs and rabbits by KRÖNING (1930). The three sets of results are in agreement in finding a strong reaction in "type" and no reaction in the white parts of white-spotted and albino animals. In the dark extremities of albino guinea pigs and in the intermediate combinations $c^k\ c^k$ and $c^k\ c^a$,[2] both in the presence of *E*— and *e e*,[3] KRÖNING claims that the reaction was as strong as with *C*—. KRÖNING further claims that in chinchilla rabbits the agouti-banded hairs reacted with full intensity irrespective of whether the bulbs were in the sepia-producing or the white-producing phase. SCHULTZ states that yellow rabbits gave a weak reaction. KRÖNING reports no difference in guinea pigs between black and red, nor between pale sepia and pink-eyed red, and suggests that, since comparisons with black are difficult, SCHULTZ may have been mistaken. The present results, indicating a slight reduction in *e e* combinations as compared with *E*— combinations, stand in an intermediate position. KRÖNING claims that substituting *p p* for *P*— reduces the reaction in the presence of either *E*— or *e e*. The present results agree with this in direction in so far as the basal layer is concerned, but in the hair bulbs there was, as has already been stated, no evidence of a reduction either in the combinations studied by KRÖNING or in many others. SCHULTZ found that brown rabbits reacted slightly less strongly than black (this was confirmed by KRÖNING), an effect which is in agreement with the present results as far as they go. The factor *f* has not been studied by earlier workers.

KRÖNING does not describe the method which he used for measuring the reaction, and such differences as exist between his results and those

[2] KRÖNING used the symbol c^H for WRIGHT'S c^a, and his symbol c^a probably corresponds to WRIGHT'S c^k. For convenience in the above discussion the symbols have been changed to match WRIGHT'S.

[3] In some cases KRÖNING used skin from the red areas of tortoise-shells, $e^t\ e^t$ (WRIGHT'S $e^p\ e^p$), instead of from self reds, *e e*, but since it is not always clear which of the two was used, only *e e* is written throughout this discussion.

reported here are seen to be differences in degree rather than direction. His final conclusions are that: (a) apart from the effect in $c^a\ c^a$ (which he does not attempt to explain) there is no unequivocal relation between dopa reaction and the albino series; (b) the quantity of dopa enzyme present depends on the *P* factor; (c) the natural chromogen is determined by the gene *E*, and the lack of chromogen in *e e* is replaceable with dopa. Some of the observations on which these hypotheses are based have, as already noted, not been confirmed by the present study. Results obtained from gene combinations not available in KRÖNING'S work have a further bearing on his conclusions and will be discussed in the next section of this paper.

Physiological genetic interpretations

Since the dopa reaction leads always to a black or sepia end-product, whereas two apparently qualitatively different pigments, sepia and yellow, are produced in guinea-pig hair, it could hardly be expected that the effects of the genes on the dopa reaction would provide a complete explanation of the physiological genetics of both sepia and yellow pigment formation. From the color of the dopa reaction, and from KRÖNING'S conclusions, a parallelism of the reaction and the grades of sepia pigment was expected. It is surprising to find that the results in the hair bulbs show no such parallelism, but do indicate a close agreement of degree of reaction with grades of yellow pigment. These conclusions will now be considered in detail.

On the simplest interpretation of KRÖNING'S conclusion it was expected that the hair-bulb reaction would, throughout both *E*− and *e e* combinations, agree with the order of effect found in the sepia pigments of the coat. This has not proved to be the case. No combination with $c^r\ c^r$ has shown a definite reaction. This holds even in the presence of *E*−. For example, *C*−*b b* and $c^r\ c^r\ b\ b$ have almost identically the same average grade of coat color, yet $c^r\ c^r\ b\ b$ gave either no reaction or, at most, one very much less than that given by *C*−*b b*. Arguing further against a correspondence with the sepia pigments is the lack of effect of *p* upon the reaction and the marked effect of *f*. This is a complete reversal of the effects of these genes on the natural sepia pigments.

The reaction of the hair bulbs, in *e e* combinations at least, does, however, agree closely with the grades of yellow pigment found in these combinations. The lack of effect of *p*, and possibly *b*, and the marked effect of *f*, are paralleled in the grades of natural yellow pigment. Even more striking is the agreement in the order of reduction with the albino series. Reference has already been made to four significantly different levels of reaction, *C*−, c^k or $^d\ c^k$ or ^{d}, c^k or $^d\ c^r$ or ^{a} and c^r or $^a\ c^r$ or ^{a}, found in the com-

binations *e e* and *e e p p*. These levels correspond to the natural colors, red, yellow, cream and white, found with the same four groups of albino alleles in the presence of *e e*. On the assumption on which the present work was undertaken, that the dopa reaction indicates the presence of an oxidizing enzyme system, the simplest interpretation of the above results is that gene replacements at the *C* and *F* loci alter the amount of yellow pigment produced by affecting the concentration or activity of the enzyme system present.

As far as they go, results in the hair bulbs in the presence of *E* indicate that the reaction, in its threshold and order of effect with the *C*− series, its reduction by *f* and lack of reduction by *p* much more nearly parallels the intensity of yellow in the corresponding *e e* combinations than it does the intensity of the sepia pigments actually present in the *E*− combinations tested. There is, however, an apparently significant intensifying effect of *E* over *e*. These facts, while interesting, do not, at the present time, provide a simple clue to the natural biochemical effect of gene replacement at the *E* locus or, related to this, the order of effect of the remaining genes in *E*− combinations. It appears likely that this part of the problem will not be solved by the dopa method alone.

Turning now to the epidermis, it has already been pointed out that the natural pigmentation in this location is quite different from that in the hair. It is, therefore, apparent that the final effects produced by interaction of the color genes depend upon regional factors. In the two paragraphs following it will be seen that the dopa results furnish evidence on both the physiological genetics of the epidermis and the nature of the biochemical difference in the two locations, skin and hair.

From table 3 it is evident that in the albino series the threshold for the dopa reaction is higher than that for pigment formation, the reaction occurring, in fact, only with *C*−. It may be that a weak reaction occurred with lower alleles, but was obscured by the natural pigment, while a reaction with *C*− was strong enough to be clearly visible. It has already been stated that the evidence of a difference in natural pigmentation between *E*− and *e e* combinations is not regarded as sufficient. Apart from the apparent threshold difference with the *C*− series, and a possible lack of agreement in the effect of *E*, the reaction parallels the natural pigmentation in the epidermis, and, in view of the fact that there is little or no reaction in all *p p* combinations, it cannot be said to parallel any kind of pigment in the hair. The simplest physiological interpretation is that, in the basal layer of the epidermis, the lower alleles of the *C*− series and *p* produce their effects, at least in part, by reducing the concentration or activity of the enzyme system present.

Regarding the difference between epidermis and hair bulbs, it is seen

that the different effects produced by the same gene combinations in the two regions are paralleled, in *e e* combinations at any rate, by differences in the dopa reaction. It follows, again on the simplest interpretation, that one biochemical result determined by regional factors is a difference in the enzyme systems active in the two locations.

So far the effect on the dopa reaction of the gene *s* has not been considered either in hair bulbs or epidermis. In white-spotted animals there is clearly a failure of enzyme in the white spots, but whether this means a change in the physiology of the melanoblasts or actual absence of these cells is not shown.

SUMMARY

In an attempt to elucidate some of the processes by which color genes produce their effects the following results were obtained:

1. Natural pigmentation in the epidermis is similar to that, observed by WRIGHT, in the eye, both in its quality and in the order of effect of the genes studied.

2. The technique used in treating frozen sections of skin with a buffered solution of dopa produced adequately uniform results within each genotype.

3. The effect of gene *s* was shown by the absence of any dopa reaction in the white area of a white-spotted animal that gave a full reaction in the black area. Combinations with *e e* gave, in the hair bulbs, slightly, but significantly, less reaction than the corresponding combinations with *E*−, while there was no difference between the reactions in the basal layer of these two combinations. As far as the evidence goes there was no difference in reaction between the two phases of color production found in agouti, *A*−, and the reaction obtained agreed with that found in *a a*. As compared to a strong reaction with *C*− the lower alleles of the albino series gave no reaction in the basal layer and reduced reaction, or no reaction, in the hair bulbs. In the hair bulbs four significantly different levels of reaction were shown: none with $c^{r \text{ or } a}\ c^{r \text{ or } a}$ combinations, weak with $c^{k \text{ or } d}\ c^{r \text{ or } a}$, medium with $c^{k \text{ or } d}\ c^{k \text{ or } d}$ and strong with *C*−. Combinations with *f f*, as compared with *F*−, gave no difference in reaction in the basal layer, but significantly less reaction in the hair bulbs. Combinations with *p p* showed almost no reaction in the basal layer, while the hair bulbs gave as strong a reaction as those in the corresponding *P*− combinations. If the gene *b* had any effect in the few combinations in which it was tested, it can only have been a slight reduction.

The following physiological genetic interpretations are made:

1. Gene replacements at the *C* and *F* loci alter the amount of natural yellow pigment produced in the hair by affecting the concentration or activity of the enzyme system present.

2. In the epidermis the lower alleles of the $C-$ series and p produce their effects, at least in part, by reducing the concentration or activity of the enzyme system present.

3. There is a difference in the enzyme systems active in pigmentation in the two locations, skin and hair.

4. There is a failure of enzyme in the white areas of $s\,s$ animals.

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