GENETIC STUDIES OF A DARKENING OF THE DORSAL COAT IN AGOUTI MICE.

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A Thesis presented to the University of New Zealand in partial fulfilment of the requirements for the degree of Master of Agricultural Science.

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D. A. E V A N S

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TABLE OF CONTENTS

LIST OF FIGURES

- - - - - - -

INTRODUCTION

It is the candidate's contention that an understanding of the mechanisms of gene action and interaction at the physiological level, is a necessary basis for a fuller understanding of the principles and problems of animal breeding. On this point, Rae (1958) states ... "population genetics deals essentially with genes and final phenotypes, but has little to say about the vast developmental gap between the two". However, opportunities for physiological studies of simple Mendelian traits in farm livestock are few and far between, while the expense of such studies is normally prohibitive.

Consequently, physiological genetic studies are normally carried out on small animals, especially the mouse. Gruneburg (1952) has reviewed the use that has been made of this mammal for genetic studies of all types.

The steadily increasing number of reported mutants affecting hair growth and pigmentation in the mouse provide excellent experimental material for studies on gene action, while their pleiotropic effects provide material for studies on the physiology and development of hair growth. As Chase (1954) has pointed out, in the one hair follicle can be seen all aspects of cell development, growth, division, and death at different stages of the hair cycle. The hair itself provides a permanent record along its length of the changes that have occurred in the hair follicle during the period of hair growth (Russell, E.S. 1946).

The studies to be described concern attempts to investigate yet another of these mouse hair mutants.

REVIEW OF LITERATURE

1. THE HAIR

A. DEVELOPMENT AND GROWTH

Dry (1926) has described the major hair types of the mouse, and the development and succession of hairs in relation to the age of the mouse and position on the body. Considering hairs of the first pelage from the mid-dorsum, this author suggested a classification of hairs according to total length, the number of constrictions, and the maximum number of medullary cells (septules) in cross section. Table 1 has been constructed from information in Dry's paper.

Intermediate hair types exist, but grow from less than 1% of the mid-dorsal follicles. The work of Falconer Frazer and King (1951), to be discussed later, suggests that at least part of this classification is real, and not a subjective division of a continuous series.

Table 1

Hair Type.	Length	Constrictions	Septules	%
Monotrichs or Type A.	.9 - 1.0 cms	0	2	2
Awls or Type B.	.6 - .7 cms	0	3-4	14
Auchenes or Type C.	.6 - . 7 cms	1	2	
Zigzags or Type D.	.4 - .7 cms	3-4	1	82

Dry (1926) has divided hair development and growth into four stages. Anagen covers the growing period, from the beginning of follicle invagination until completion of hair growth. Chase (1951) divided the new anagen stage initiated after plucking, into six separately recognizable

stages, and has shown that the pattern of development after plucking is identical with the pattern of development of the first pelage, after birth. These six stages of anagen are described as follows.

Anagen 1 appearing 1-2 days after plucking - cell division of the germ plate, which is formed in the Malpighian layer.

Anagen 2 (2-3 days) - down-growth of the germ over the papilla.

Anagen 3 (3-4 days) - papilla cavity and bulb matrix formed; melanin produced.

Anagen 4 (6 days) - cortex and medulla formed; tip of shaft near sebaceous gland; pigment granules in matrix.

Anagen 5 (8 days) - tip at skin surface.

Anagen 6 (8-17 days) - period of hair growth.

At the completion of hair growth, the follicle passes into a short stage of regression which Dry (1926) has termed Catagen. Chase (1951) describes the events of this stage which normally occupy a period of two days, as follows. The cessation of matrix-cell proliferation in the bulb - cessation of pigment production - formation of a hollow brush of kerotinized cells at the lower end of the compact cortex (the "club") - rounding up of the papilla cells into a ball - shrinking of the club region and movement away from the germ layer towards the skin surface.

On the completion of catagen, at approximately 19 days post partum the follicle enters into a resting stage which lasts until the beginning of the anagen of the next pelage. This resting stage has been termed Telogen by Dry (1926), and marks the end of the first hair cycle. The following hair generations follow a process similar to the first, except that in anagen 1, the new papilla is formed from the regeneration of the dormant hair germ, and not from a downgrowth of the malpighian layer.

B. Time relations.

Time and space relations of successive hair cycles, have been described by Dry (1926), and more recently by Borum (1954). The following discussion is confined to the first pelage.

Gruneburg (1943) using a mixed strain of mice which he describes as "extremely heterogeneous", found easily recognizable hair follicles on the sides of 14 day old embryos, and on the dorsum at 15 days. This is supported by the work of Falconer Frazer and King (1951) in comparing normal and crinkled mice. Mice homozygous for the crinkled gene (cr/cr), which is recessive to the normal allele, showed an absence of monotrichs and zigzags from the coat. In contrast to the normal mice studied, follicle initiation began at 18 days instead of 14 days, and no new follicles were added after birth. These facts prompt the authors to state: "The absence of guard hairs and of zigzags from the coat of the crinkled mouse, and the resemblance of the crinkled hairs to awls of normal mice constitutes strong evidence in support of the hypothesis that guard hairs are produced by follicles that form before 17 days, awls by those that form between 17 days and about the time of birth, and zigzags by those that form chiefly after birth". Dry (1926) states that zigzag follicle initiation continues to about 7 days post partum on the dorsum and 9 days post partum on the venter.

Borum (1954), using albino mice of the St/Eh strain found the first hairs appearing on the head back and neck at the age of 2-3 days after birth and on the venter at 4-5 days. The entire back is covered by 8-10 days, and the growth of the first pelage is complete at an age of about 12 days. The pattern described by Dry (1926) studying entire agouti skins is virtually the same, although the respective ages are later. This author describes telogen appearing first on the head at 15 days of age, and on the venter at 17 days, with the last area to complete growth being the sacrum at about

19 days.

Relatively little is known regarding factors controlling or affecting time relations of the first hair cycle. The speed of the second hair cycle is affected by sex; female cycles starting later and finishing later, as well as being of longer duration (Borum, 1954; Cockren 1959). Blood supply to the follicle, body weight at weaning, rate of body growth, and age at which the cycle is initiated, may all be factors affecting or correlated with, length of duration of the second hair cycle, (Cockrem 1959, 1962).

II. THE PIGMENT

(A). Biology.

All pigments found in mouse hairs are granular melanins of two types, (Gruneburg 1952), whose properties are illustrated in Table 2, extracted from Table 1 of Fitzpatrick et al. (1958)

Table 2

	Eumelanin	Phaeomelanin
Colour	Brown-Black	Yellow
Precursor	Tyrosine	Tyrosine (Tryptophan)?
Shape	Oval-Round Granules	Round Granules
Chemical Properties	Insoluble in Dilute Alkali	Soluble in Dilute Alkali

The biological unit of melanin production is the melanocyte cell. Rawles (1947) has shown that in the mouse embryo, melanoblasts originate from the developing neural crest, and migrate by as yet unknown mechanisms to all regions of the mouse body by the 12th day of gestation. According to the tissue and biochemical environment in which the melanoblast is located, it will either remain colourless or start to produce melanin, as

has been shown by Weiss and Andres (1952) working with chick embryo homogenate transplants.

Chase (1951) in reviewing the process of melanoblast incorporation into the follicle, states that the melanoblasts are found on the basement membrane of the surface epidermis, with their cell bodies lying between the cells of the malpighian layer. Chase and Smith (1950) have shown that some of these melanoblasts which are unpigmented, are included in the original follicle invagination and thus become interspersed with cells of the follicle germ plate.

The now active melanocytes become concentrated in the top half of the bulb matrix, where presumptive hair cells have low mitotic rates, (Chase 1951), and by a device not yet clear, they innoculate melanin granules into the stream of passing cortical and medullary cells. Evidence from work with the electron microscope (Montagna 1961), suggests that the recipient cells actually phagocytose the pigment-bearing tips of the melanocyte's dendrites.

Just before the onset of catagen, the melanocyte regresses and melanin production ceases (Chase 1951). The origin of melanocytes for the next hair cycle is as yet unknown, although the above author suggests the following possibilities.

(i) Regeneration of the former melanocytes.

(ii) Replacement from melanoblasts in the epithelium.

(iii) Development of melanoblasts from stem cells.

(iv) Replacement from possible dermal sources.

Chase (1960) states that the bulk of the evidence supports (iii) above. However, the possibility of a dermal source should be considered in light of the recent results of Nay (1956), working with Naked and crinkled

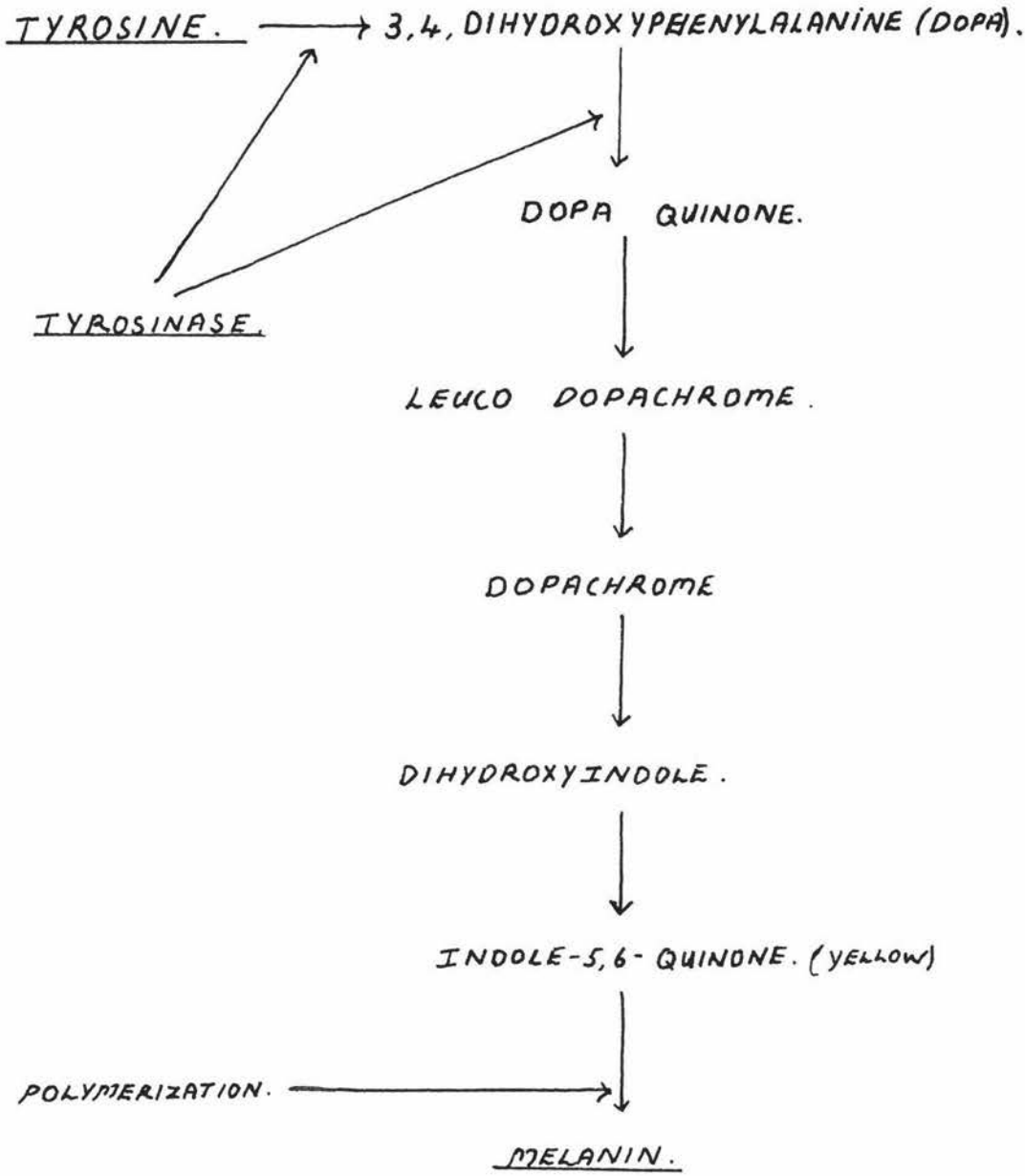


FIG. 1.

PATHWAY OF MELANIN SYNTHESIS.

mice, which enabled him to conclude that dermal mast cells below the follicle bulb may have a function in melanin production.

(B) Biochemistry

1. Chemistry of Melanin Synthesis.

This subject has been reviewed by Fitzpatrick et al (1958). The principal precursor of black melanin is tyrosine, while the production of yellow melanin may involve tryptophan as well. The metabolism of these two compounds has been reviewed diagrammatically by Harper (1959). All melanins are formed by the action of a group of copper-containing enzymes (phenolases) which catalyse the conversion of phenols and diphenols to the corresponding quinones.

The path whereby tyrosine is converted to black melanin was first elaborated by Raper (1928) using the Meal-worm (Tenebrio molitor), and was confirmed by Mason (1948) using malignant mouse melanoma. This pathway is shown in Figure 1, taken from Fitzpatrick et al (1958).

The melanin polymer is attached to a protein through its quinone linkages, and recent work of Mason (1959) suggests that at least in DOPA melanin, the sulphydryl groups of the protein are involved.

It is now clear (Lerner 1949) that the enzyme tyrosinase catalysing the two separate steps,

Tyrosine ---------> DOPA

and DOPA ----------> Quinone,

is not a mixture of phenolases, but a single enzyme. Mason et al (1955) have shed some light on the question of how one enzyme might catalyse two separate reactions. The initial step in the reaction is the reduction of tyrosinase from the cupric to the cuprous state, by the action of DOPA, which must thus be considered as an activator of tyrosinase as well as

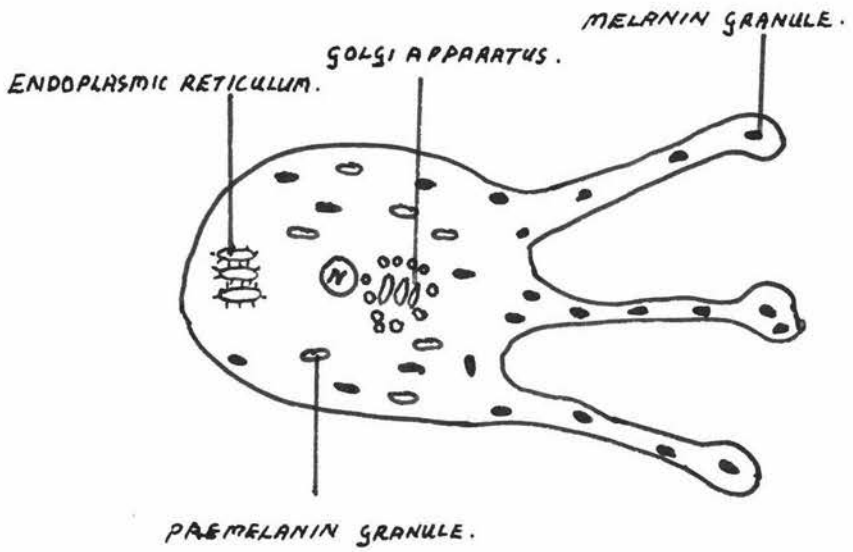


FIG. 2.

DIAGRAMMATIC REPRESENTATION OF A TYPICAL MELANOCYTE.

being a substrate for the enzyme. Hence the rate of oxidation of tyrosine appears to be related to the rate at which DOPA may be enzymatically oxidised to DOPA-quinone. Thus there exists an inverse relationship between the induction period in the oxidation of tyrosine by tyrosinase and the concentration of the enzyme.

2. The Melanocyte

The metabolic unit of melanin formation in the melanocyte is the melanin granule, (Birbeck et al 1956) which consists of a protein matrix. This matrix contains tyrosinase and at least two other enzymes, cytochrome oxidase, and succinic dehydrogenase. Birbeck and Barnicot (1959) have carried out electron microscopy examinations of the melanocyte, and suggest the structure of the cell to be as that shown in Figure 2, which is taken from their paper. According to these authors, the protein matrix of the premelanin granule, which contains the tyrosinase, arises from small vesicles in the golgi zone. Melanin is deposited upon the protein which results in a steady decrease in detectable tyrosinase activity.

3. Melanin Synthesis in Relation to the Hair Cycle.

Two principle techniques have been used in studies of the tyrosinase activities of mammalian hair roots in vitro.

(1) Using radioactive tyrosine-2-C^{14} as a substrate for tyrosinase, followed by the estimation of enzyme activity from the intensity of radioautographs (Fitzpatrick and Kukita 1956).

(ii) Using 3-4-dihydroxyphenylalanine (DOPA) as a substrate and measuring enzyme activity by the intensity of the black pigment produced (Russell.E.S. 1948).

Kukita (1957) has followed the changes in enzyme activity with the hair growth cycle in C_{57} mice, using both methods.

Table 3 has been prepared from the results obtained by this author.

Table 3

Stage	Pigmented Melanocytes Present	Tyrosinase Activity	DOPA-oxidase Activity.
Anagen 1.	-	-	-
Anagen 2.	-	-	-
Anagen 3.	+	+	++
Anagen 4.	+	++	++
Anagen 5.	+	+++	+++
Anagen 6.	+	+++	+++
TELOGEN	-	-	-

Tyrosinase activity appears simultaneously with the appearance of pigment in Anagen 3, and disappears with the cessation of pigment production in Telogen. It is not known whether the increase in enzyme activity over the later stages of Anagen is due to melanocyte proliferation, increased activity within the melanocytes, or the development of new melanocytes. It is confusing in this respect in that Montagna (1956) states that melanocytes are capable of undergoing mitosis, ~~which~~ while Chase (1958) suggests that if mitoses do occur, then they do so only in the immature stages.

Which factors operate in controlling the rate of melanin synthesis in vivo during the hair cycle are unknown. Rothman (1954) has suggested that the level of -SH ~~in~~ groups in the bulb may be an important factor. In early Anagen when there is a high concentration of these sulphydryl groups in the bulb, tyrosinase activity may be inhibited by the action of these groups on the copper prosthetic group of the enzyme. This inhibition is removed in later Anagen stages when the level of free -SH groups is known to decline. However, Montagna (1956) points out that -SH groups may not be detectable in the bulb during later Anagen, but are likely to be present due to the presence of glutathione which is required for cell division in the matrix, and is a tyrosinase inhibitor.

The possibility of an inhibitor which changes in concentration with phases of the hair cycle is an attractive hypothesis (Montagna 1956, Fitzpatrick et al 1958), for melanin production ceases abruptly just before the end of hair growth, as if the melanocytes were sensitive detectors of the end of the growing period.

4. Yellow Pigment

Both tyrosinase and DOPA-oxidase activities (Foster 1951, Russell 1948), have been detected in phaeomelanin hair follicles of man, mice, and guinea pigs, thus these follicles might oxidise tyrosine and DOPA in vivo. However, as will be discussed later, DOPA-oxidase activities in vitro are more closely related to the genetic "potential" amount of phaeomelanin, than of eumelanin, (Russell 1948), even though the pigment formed is black. This apparent discrepancy has led Haldane (1954) to comment that "Whatever dopa oxidase is doing in mouse skins, it is not oxidising 3-4-dihydroxyphenylalanine, though it is very probably concerned in some phase of production of yellow pigment from a precursor". In discussing this problem Fitzpatrick et al (1958) suggest that three points must be taken into account.

(i) In the agouti hair, one follicle synthesises both types of pigment according to the stage of hair growth, and thus the type of pigment in the coat must depend on local conditions within the follicle.

(ii) The single gene substitutions e/e in the guinea pig and $A^y/+$ in the mouse cause a clear cut change to yellow pigment with no intermediate colours formed, which is strongly suggestive of a single enzymatic step.

(iii) Phaeomelanin production must involve the activity of tyrosinase but without implicating tyrosine as the pigment precursor.

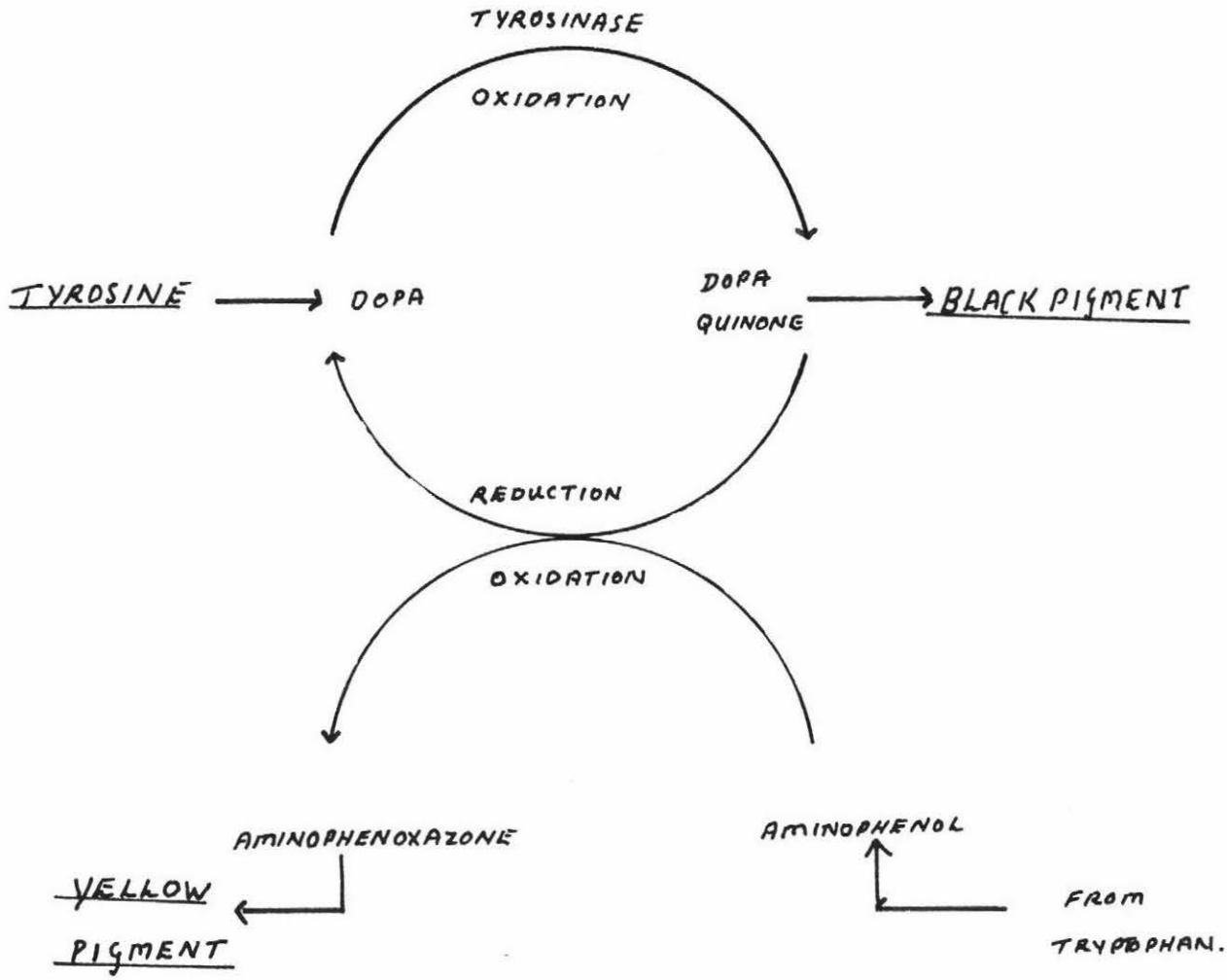


FIG. 3.

A POSSIBLE YELLOW-BLACK PIGMENT "SWITCH" MECHANISM.

More positive evidence has been obtained by Brunet et al (1957) and Butenandt (1956) who both obtained yellow pigment by incubating red hair roots and red silk-worms respectively, with a mixture of DOPA, and tryptophan metabolites (such as 3-hydroxykynurinine and 3-hydroxyanthranilic acid). The reaction depends on the conversion of DOPA to DOPA-quinone in the presence of tyrosinase, and the non-enzymatic oxidation of 3-OHkynurinine to xanthommatin by DOPA-quinone, which is reduced back to DOPA. Fitzpatrick et al (1958) speculate that such a mechanism (as shown in Fig. 3) might explain the black-yellow pigment production switch, as a result of the presence or absence of an O-aminophenol in the follicle, and is also compatable with the fact that phaeomelanic hair follicles contain tyrosinase.

III. Background Genes

The following discussion has been confined to the agouti series of coat colours and the semidominant mutant white (Mi^{wh}), which were the background genes upon which the darkening effect under study was investigated.

A. Agouti Locus.

1. Description of Alleles

The agouti series of alleles has been reviewed by Gruneburg (1952), and at that time was thought to consist of five alleles labelling the fifth linkage group of the mouse. These are described in Table 4 taken from Gruneburg (1952) and are shown in decreasing order of dominance.

Table 4

Phenotype	Symbol
Yellow	A^y
White-bellied agouti (Light-bellied)	A^w
Grey-bellied agouti	A^+
Black-and-tan	a^t
Non-agouti (black)	a
Extreme non-agouti	a^e

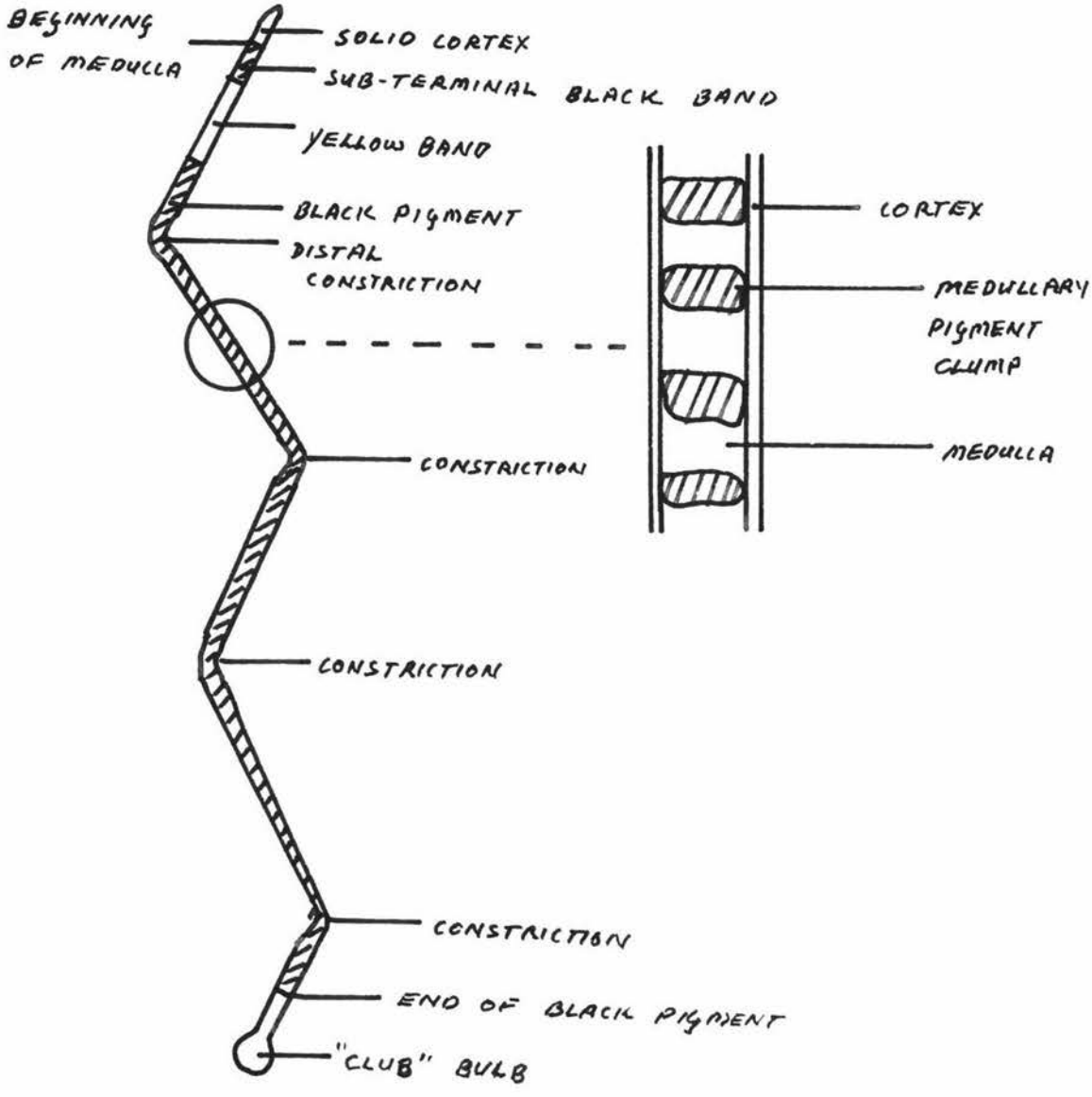


FIG. 4.

AN AGOUTI ZIGZAG HAIR IN THE CLUB STAGE.

(NOT TO SCALE)

Yellow ($A^y/+$), the top dominant of the series, is lethal when homozygous but was not available for this study. Extreme non-agouti (a^e/a^e), has been reported by Hollander and Gowen (1956) and was also not available for this study.

The following is a brief description following from Gruneburg (1952) of the four alleles at the agouti locus used in the present investigation.

(a) White-bellied agouti.

On the dorsal surface, the hairs consist of a black base, yellow subterminal band, and black tip (agouti pattern) giving the coat a brownish-grey appearance. A typical agouti zigzag hair is shown in Fig. 4. The ventral hairs have a lighter coloured base, with wider light tips, giving a light cream or white appearance, or a cream-yellow appearance, according to the presence or absence of as yet unknown modifying genes (Little 1916, Falconer 1947). The difference in colour between the dorsal and ventral surfaces is sharply marked, and often accentuated by a scattering of orange coloured hairs along the dividing line.

(b) Grey-bellied Agouti

The dorsal surface is the same as that of white bellied agouti, but the ventral surface is grey, with no sharp demarcation on the flanks.

(c) Black-and-tan

Hairs of the dorsal surface are completely black, while the ventral hairs are yellow with a dull grey base, giving the ventral surface an orange-yellow colour with perfectly sharp borders on the flanks.

(d) Non-Agouti

Both dorsal and ventral hairs are completely black.

All the above four phenotypes have a small tuft of yellow or white hairs behind the ears.

The dominance relations of these four alleles are shown in Table 5, taken from Table 14 of Gruneburg (1952).

Table 5

Genotype	Phenotype	
	Dorsal	Ventral
A^W/A^W, A^W/A^+, A^W/a^t, A^W/a	Agouti	White
A^+/A^+, A^+/a	Agouti	Grey
A^+/a^t	Agouti	Light
a^t/a^t, a^t/a	Black	Yellow
a/a	Black	Black

Gruneburg (1952) points out that A^+ appears to be dominant over a^t on the dorsal surface, but a^t is dominant over A^+ on the ventral surface.

Dry (1928) has described the agouti pattern of the wild mouse in relation to its hair type. For hairs of the same pelage taken from the mid-dorsum, this author's results can be summarised as follows:

Monotrichs - Black throughout

Awls - Yellow bands may or may not be present, with the larger awls tending to be non-banded.

Auchenes - No difference in total hair length between black and banded auchenes.

Zig Zags - Length of band increases with length of hair, with virtually all zig zags being banded.

On the venter, the situation is reversed, with awls and auchenes showing a direct positive relationship between hair length and yellow band length, while zig zags show the inverse relationship.

2. PHYSIOLOGY OF THE AGOUTI ALLELES

Russell, E.S. (1946, 1948) carried out a histological examination of the pigment characters of hairs, from mice representing thirty-six colour genotypes. The following seven variable attributes were measured at a magnification of 1800X.

(i) The number of granules per medullary cell.

(ii) The number of granules per unit volume of cortex. The unit volume of cortex was that volume estimated to be equal to the volume of an average medullary cell.

(iii) The tendency towards a distal arrangement of granules within the medullary cells, graded as "none", "slight" and "cap".

(iv) The diameters of medullary pigment granules, each granule being measured along its major axis.

(v) The shape of medullary pigment granules, graded as "long-oval", "short-oval", "round" and "irregular".

(vi) The tendancy towards the clumping of granules, graded as "loose flocular" and "granular-clumps".

(vii) The colour of the granules, as matched against Ridgway's Colour Standards.

Attributes (i), (ii) and (iii) were measured on whole mounts at successive ten microscope field intervals down the hair, starting at the fourth field from the tip of the hair. Attributes (iv) to (vii) were measured on cross-sections which had been cut somewhere between the fifteenth and forty-fifth fields.

This author's results enabled her to show that variation in any one

of the above attributes could be reflected in differences in coat colour; i.e. each of these variables is under genetic control of known coat colour genes. By comparing results at successive levels down the hair this author was also able to show that the agouti series of alleles have no effect on the above characters other than to direct pigment production into either phaeomelanin or eumelanin. The yellow bands of A^w/A^w genotypes contain the same type and quantity of phaeomelanin as in the corresponding region of the comparable A^y/a genotype, while the type and quantity of eumelanin in the rest of the hair is identical with that in the corresponding region of the comparable a/a genotype.

Russell L.B. and Russell W.L. (1948) have investigated the DOPA-oxidase activities of the major mouse coat colour genotypes, and have compared their results with the quantitative histological pigment measurements obtained by Russell E.S. (1946, 1948). To determine the DOPA-oxidase activities, skin samples were taken mid-dorsally from the lumbar region of 6-7 day old mice. After fixing in formalin, sections were cut on a freezing microtome and incubated in a buffered DOPA solution. The resulting sections were mounted and the amount of DOPA-melanin in the hair follicles was estimated by comparison with standard slides representing six levels of intensity. Estimates of the DOPA-oxidase activity obtained in this manner for each genotype were expressed as a percentage of the wild-type (CCBBPPDD) value for both a/a and A^y/a backgrounds.

To obtain a value for comparison with the DOPA-oxidase activities, an estimate proportional to the total volume of pigment output from the follicle was derived for each genotype, from the data of Russell, E.S. (1946, 1948). Only data from the 20th, 30th and 40th microscope fields were used in obtaining this estimate, as this region of the hair corresponded to that being produced by the follicle when the DOPA-oxidase activities were

being measured. The estimates of total volume of pigment obtained in this way for each genotype were expressed as a percentage of the wild-type value for both a/a and A^y/a genotypes.

Although qualitatively, the pigment produced by the DOPA reaction resembled eumelanin, there were no differences in the DOPA activities of corresponding A^y/a and a/a genotypes. Further, changes in DOPA-oxidase activities induced by gene substitutions, were directly related to changes in the volume of pigment produced by those substitutions, only in A^y/a genotypes. This situation is understood more clearly on inspection of Table 6, based on information taken from Table 1 of Russell, L.B. and Russell, W.L. (1948).

Table 6

DOPA grades and pigment volumes, both expressed as a percentage of wild-type, for various mutants on both A^y/a and a/a backgrounds.

	A^y/a		a/a	
Mutant	DOPA	Pigment	DOPA	Pigment
CCBBPPDD	100	100	100	100
bb	102	87	101	41
pp	103	96	101	16
bb dd	110	130	100	46
$c^h c^h$ pp	48	31	34	9
$c^h c^h$ dd	72	63	50	87
$c^h c^h$ bb	56	44	50	37

According to these authors, "the conclusion must be drawn that the DOPA reaction measures some phase of the yellow producing system and that this system is present in sepias as well as yellows."

The time, and site, of action of alleles at the agouti locus, have been studied in mice by Reed (1938a,1938b) and Silvers (1955, 1958), respectively. Reed (1938a) carried out skin transplants using the skin of newly born or near-term embryos. Transplants between black-and-tan and grey-bellied agouti phenotypes, and between dorsal and ventral surfaces of the same mouse, showed autonomy of the grafts in all cases. Thus the colour differences appear determined before birth. Reed (1938b) extended this work to include non-agouti with black-and-tan, both of which proved to be autonomous with each other after the embryos were 11 mm. long. This author was also able to show that host epidermal melanocyte migration took place into the donor follicles and although the skin tissue transplants showed autonomy, the migrating melanocytes did not. Silvers (1955, 1958) extended this technique to the other alleles at the agouti locus, and used Russell's (1946) pigment characters to determine whether pigment in any part of the hair came from "donated melanoblasts" of the host or from "host follicles" of the donor. Migrating pigment cells of all agouti types responded to the genetic constitution of the receiving hair follicle. Thus all genes so far studied at the agouti locus show non-autonomy of the pigment cell and autonomy of the follicle. It is the agouti genotype of the hair follicle which determines whether a melanocyte will produce eumelanin, phaeomelanin, or both.

B. DOMINANT WHITE

This semidominant mutation (symbol Mi^{wh}) was obtained by Grobman and Charles (1947) from the offspring of an X-irradiated male mouse and hence was probably induced. These authors describe the coat colours of the three genotypes as follows.

Mi^{wh} Mi^{wh} aa - indistinguishable in colour from albinos

Mi^{wh} mi^{wh} aa - grey

$mi^{wh}m^{wh}aa$ - black

These authors also studied individual hairs sampled from the mid-dorsum at 46 and 76 days of age, and hence probably represent the second and third pelages. The hairs were cleared over a period of 3-4 days in increasing concentrations of xylol in 95% alcohol, mounted on slides and studied under the projection microscope. Tracings were made of 100 micron lengths at a position proximal to the second bend from the distal tip of a zigzag hair. A straight line was drawn through the centre of the medullary pigment bands, parallel to the length of the hair, and measurements of the widths of the bands were made along the line. The sums of the widths provided a measurement of pigment in the hair. The amount of pigment, expressed as microns of pigment per 100 microns of hair, ranged from 55 to 70 for $mi^{wh}mi^{wh}aa$ mice, and was reduced to 35 to 45 in $Mi^{wh}mi^{wh}aa$ genotypes. Other effects of the Mi^{wh} gene in the hetrozygous state are to cause lighter coloured tails and feet, dark ruby rather than black eyes, and occasionally a white ventral or dorsal spot. Homozygous whites show a reduction in the size of the eye, and the Mi^{wh} allele generally is accompanied by a decrease in fertility as measured by mean litter size.

Lane and Green (1950) have shown that Dominant White is an allele of Microphthalmia (Mi/Mi) and thus the original symbol Wh is now Mi^{wh}. This gene locus is located in the XI linkage group.

Markert and Silvers (1956) have examined the occurrence of melanocytes in six different body tissues of the mouse. Mice of the genotype Mi^{wh}/Mi^{wh} have no melanocytes in the choroid, hair follicle, ear skin, Harderian gland and nicitans, but do have melanocytes producing a small amount of pigment in the retina. Mice of the genotype Mi^{wh}/mi^{wh} show pigment producing melanocytes in the retina, hair follicle, ear skin and nicitans, but have no melanocytes in the choroid and Harderian gland.

Consequently, these authors suggest that the Mi^{wh} gene has its effect on the early steps in melanoblast differentiation by controlling or altering the tissue environment of the melanoblast.

IV. UMBROUS GENES

Gruneburg (1952) has reviewed the small series of genetic modifiers which operate to cause a darkening of the dorsal coat in mice. Modified yellows or "sables" are caused by the presence of variable numbers of dorsal hairs containing cortical eumelanin in the coat of yellow ($A^y/+$) mice. The "sables" form a continuous series in colour from dingy yellow through to glossy black, with increasing numbers of the black pigmented hairs. The intensity of colour changes with successive moults, and such mice are usually darkest at about 3 weeks of age. Such modifying factors have collectively been termed "umbrous" genes by Barrows (1934).

Dunn (1920, reported Gruneburg 1952) has shown that these genes were independant of the agouti series. On the agouti background, their effect varied from the production of a streak of dark pigmentation down the mid-dorsal line, to an almost completely black colour over the entire dorsal surface. The darkening effect of these genes was the same in a/+ and +/+ genotypes.

Barrows (1934) showed that at least some "umbrous" genes were effective only in the a/+ genotypes, and as such act as dominance modifiers of non-agouti.

Mather and North (1940) have studied the action of an isolated "umbrous" gene (symbol *u*), which darkened the agouti coat from the eyes to the tail along the mid-dorsal line, and which reduced the length of the sub-terminal yellow hair bands in this region. The gene behaved as a semi-dominant, and its effect was slightly enhanced in a/+ genotypes compared with +/+ genotypes. The gene has no visible effect on an a/a genetic background, and caused a "sable" appearance in $A^y/+$ genotypes. To determine

any agouti-"umbrous" interaction, these authors studied sets of prepared skins taken from mice at the ages of 14 days and 6 weeks. The prepared skins were graded more easily by eye than were the living animals. These skins were given numerical values believed to represent the darkening effect. The results were shown in Table 7, taken from Table IV of Mather and North's paper.

Table 7

Relative Lightness of the Hair.

	A^+A^+	A^+a	aa
UU	10	8	0
Uu	11	10	0
uu	12	11	0

Although the younger set of skins were darker, the 6 weeks set agreed in having the same interaction. The dominance of A^+ over a, appeared to be incomplete when prepared skins were studied.

Robinson (1959) has studied another "umbrous" gene (symbol U) isolated as a "sable". This gene behaved as a simple dominant and segregated as an "umbrous" gene on the agouti background also; although "umbrous" agouti and wild-type agouti phenotypes could not be distinguished as easily as "sable" and yellow. This author pointed out the near polygenic nature of "umbrous" and the possibility of overlapping "umbrous" agouti and agouti phenotypes.

The appearance of the "umbrous" phenotype was variable and no obvious differences could be seen between A^y/a^t vs A^y/A^w and A^w/at vs A^w/A^w genotypes in combination with both U/U and U/+, although prepared skins were not studied. "Umbrous" was apparently inherited independently

of agouti and probably of sex; although the χ^2 for the overall (four matings) independant assortment of *u* with sex was 4.16 which just exceeds the 5% level of significance. This "umbrous" gene had no visible effect on belly colour in either yellow-bellied or grey-bellied agouti phenotypes.

Lane and Green (1960) have reported an "umbrous" gene which caused a strong reduction in size of the yellow agouti band in hairs along the mid-dorsal line, a lesser reduction on the lateral hairs, while the ventral hairs were almost solid grey with no yellow ticking. The ears and tail were more deeply pigmented than is normal. This gene behaved as a simple recessive and has been termed mahogany (symbol mg) by these authors. The mahogany phenotype was recognisable on a non-agouti phenotype by the absence of white hairs from behind the ears, and blacker tail, feet and legs than normal non-agoutis. Linkage tests performed by these authors placed mahogany approximately 12 recombination units from the agouti locus.

Falconer (1956) has reported the appearance of a new umbrous mutant which has been named dark (symbol da). The most obvious effect on colour in $A^+/+$ and $A^y/+$ animals was that "on the back the yellow pigment is replaced by black, so that both look like a/a except on the flanks". This mutant behaved as a recessive, and gave good segregation. The homozygotes bred poorly, and showed a reduced growth rate. Falconer (1957) has shown that the dark locus is located in linkage group I.

PRELIMINARY MATINGS

I. Introduction

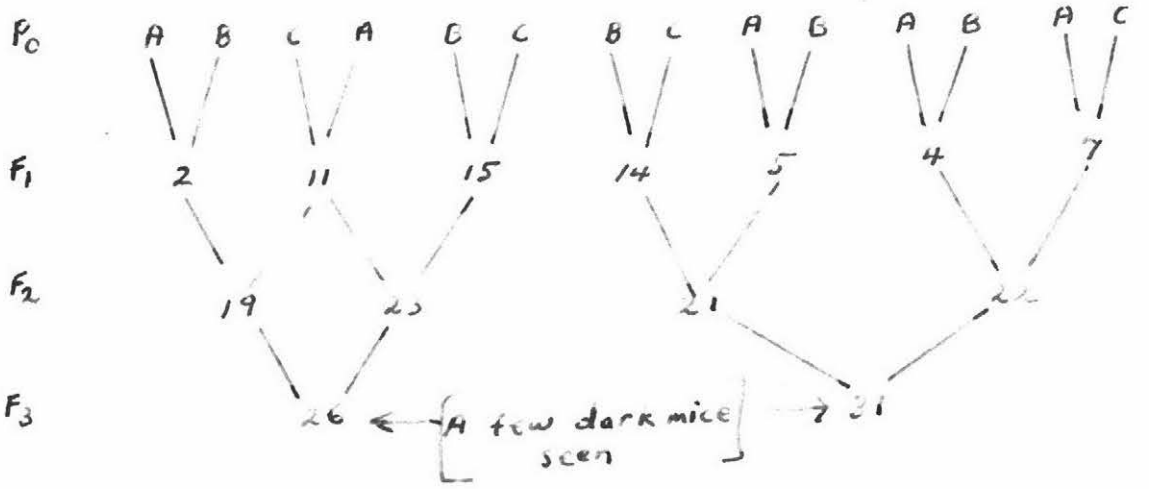
The factor under investigation was first observed in third intercross litters of body weight - tail length selection lines (Cockrem 1959). These lines were made up of crosses between the following three inbred lines:

Inbred line	A^W101	(homozygous A^W/A^W)	(A)
" "	CBA	(homozygous A^+/A^+)	(B)
" "	C_{57}	(homozygous a/a)	(C)

Designation in Fig. 5.

The pedigree, up until the first dark mice were seen, is shown in Fig. 5.

FIGURE 5

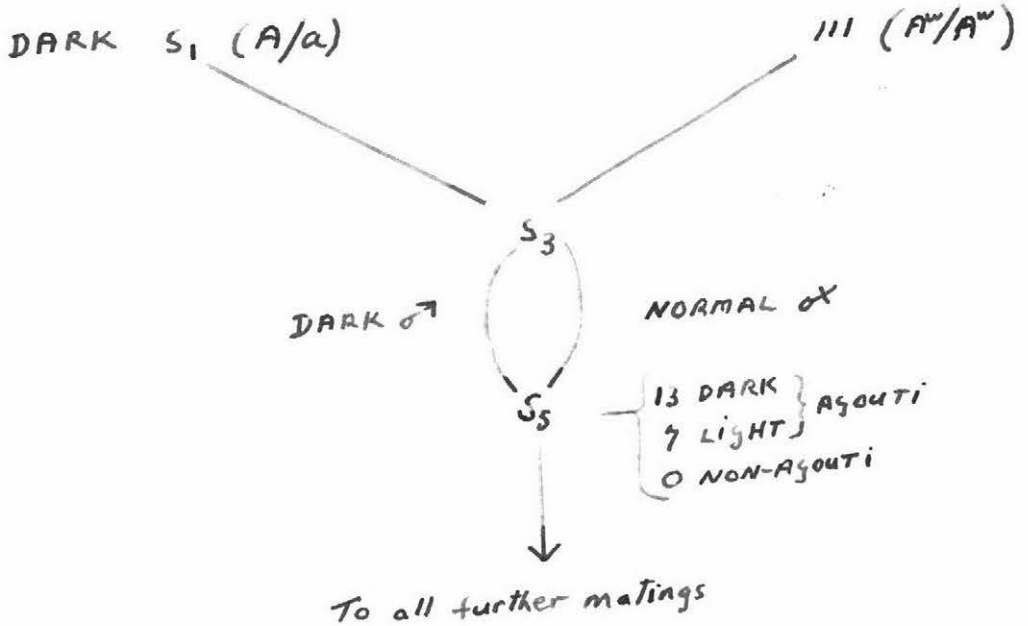


The phenotype of these animals could best be described as a darkening of the coat to an almost black colour along the mid-dorsal line of both yellow-and grey-bellied agouti mice.

Neither matings 26 nor 31 were selected for future matings but dark mice turned up in subsequent generations. Matings to investigate the genetic basis of the dark effect were started after F_5 (Cockrem pers

comm.) and the pertinant parts of this pedigree are shown in Fig. 6.

FIGURE.6.



II. Matings

In an attempt to discover the nature of the genetic situation causing the segregation of dark, Cockrem set up the following eight matings. All offspring were classified at weaning (21 days of age) into either dark or light (normal), on the visual appearance of the dorsal coat.

d_1 light (S_5) x light (S_5)

d_2 dark (S_5) x dark (S_5)

d_3 dark ♂(S_5) x light ♂(S_5)

d_4 light ♂ (S_5) x dark ♂ (S_5)

d_5 Inbred C_{57} ♂ x S_3 ♂

d_6 dark ♂(d_1) x light ♂ (d_1)

d_7 dark (d_3) x dark (d_3)

d_8 dark ♂ (d_4) x dark ♂ (d_1)

III. Results

Apart from d_5, which was a test-mating of the s_3 female, the above matings fell into two groups, according to whether or not the offspring contained some non-agouti (a/a) mice.

Matings d_1, d_3, d_4, and d_6 gave only agouti (A/+) offspring, while matings d_2, d_7, and d_8 gave some non-agouti (a/a) offspring. The results from matings d_1, d_3, d_4, and d_6 are shown in Table 9, and those from matings d_2, d_7, and d_8 in Table 11.

Table 11.

(Numbers of Mice (sexes combined) classified as Dark, Light and Non-agouti from Matings d_2, d_7 and d_8)

Mating	Dark	Light	Non-agouti	Total
d_2	19	0	8	27
d_7	?	?	1	5
d_8	4	1	3	8
Total	?	?	12	30

Unfortunately, the mother in mating d_7 died shortly after the birth of her first litter and classification of the four agouti offspring into dark and light was not possible. Due to disease, mating d_8 was terminated after classification of the first litter.

Mating d_5 gave a total of 16 mice, all of which were agouti phenotypes.

In analysing the data from Table 9, the simplest hypothesis to test is that the dark x light matings of d_3, d_4 and d_6 represent crosses of the type heterozygote x homozygote, the latter being wild-type or dark according to the dominance relations. The expectation is a 1:1 ratio for simple

monogenic Mendelian inheritance of dark:light among the offspring. To test this hypothesis, a Chi-square analysis (Mather 1938) was carried out on the data in Table 9. The light x light mating was included in the analysis on the basis that if the results differed from a 1:1 expectation, this might be shown up in the heterogeniety item. The various estimates of χ^2 are shown in Table 10, the pertinant features of which may be summarised as follows.

Table 9

Numbers of mice of each sex classified as dark and light from matings d_1, d_3, d_4 and d_6.

	Dark		Light		
Mating	Males	Females	Males	Females	Total
d_1	8	6	15	11	40
d_3	6	11	13	7	37
d_4	6	6	7	7	26
d_6	5	6	5	3	19
Total	25	29	40	28	122

Table 10

Chi-square estimates from the data in Table 9

Source	Dark vs Light	Male vs Female	Linkage	df.
d_1	3.6000	.9000	.1000	1
d_3	.2432	.1081	3.2973	1
d_4	.1538	.0000	.0000	1
d_6	.4737	.0562	.4737	1
Totals	4.4707	1.0607	3.8710	4
Combined data	1.6066	.5246	2.0984	1
Heterogeneity	2.8641	.5361	1.7726	3

P	.05	.10	.20	.30	.50	.70
χ_1^2	3.84	2.71	1.64	1.07	.46	.15
χ_3^2	7.82	6.25	4.64	3.67	2.37	1.42

(i) All estimates of χ^2 give probabilities of greater than .05. Consequently there are no statistically significant deviations from expectation in the table.

(ii) The ratio of dark: light offspring is in good agreement with the expected 1:1 ratio in matings d_3, d_4, and d_6. Mating d_1 is only just in agreement with expectation ($p = .05 - .10$) which might be the result of random sampling, classification errors or representing some ratio other than 1:1. There is no evidence of heterogeniety between the matings ($p = .30 - .50$) and consequently the data may be combined to give an overall χ_1^2 of 1.6066 which is again in agreement with the expected 1:1 ratio of dark : light ($p = .10 - .20$).

(iii) All matings give estimates of χ^2 in agreement with the expected sex ratio of 1:1 (p .30). There is no evidence of heterogeniety ($p =$ .70) and the combined data gives an overall χ_1^2 of .5246, again in good agreement with expectation ($p = .30 - .50$).

(iv) There is no evidence of linkage of dark and sex in matings d_1, d_4, and d_6 ($p > .30$) although mating d_3 is only just in agreement with the expectation of no linkage, ($p = .05 - .10$). There is no evidence of heterogeniety ($p = .50 - .70$) and the combined data is in agreement with expectation ($p = .10 - .20$).

The combined date from Table 10 was analysed in a two-way classification by the use of Chi-square (Mather 1943) to estimate any sex - colour inter-

action. The result was in agreement with the expectation of no interaction; $\chi^2 = 1.8975$ with $p = .10 - .20$.

IV. Discussion

In all the matings of dark to dark, i.e. d_2, d_7, and d_8, the offspring contained some non-agouti homozygotes (a/a), indicating that all six dark parents of these matings were heterozygotes for non-agouti. As these dark parents came from offspring of matings S_5, d_1, d_3 and d_4, and in all cases were heterozygous non-agouti, it appeared as if dark might express itself only in non-agouti heterozygotes. An inspection of the data in the body weight - tail length selection lines litter book showed that this could in fact be the case. All dark mice recorded were either non-agouti heterozygotes or could have been. In no case was a dark mouse recorded that was definitely known to be homozygous agouti.

The test-mating of the S_3 ♂ i.e. mating d_5, in which this mouse was backcrossed to a non-agouti homozygote from the C_{57} inbred line, gave no non-agouti offspring out of 16 mice bred. Consequently it is likely that the S_3 female was homozygous for agouti ($p = < .01$) and the non-agouti allele probably entered via the S_3 male. Thus the S_5 mating was probably of this type -

dark x light

S_3 ♂ (Aa) S_3 ♂ (AA)

↓

13 dark agouti

and 7 light agouti

Again, the dark S_3 ♂ was a non-agouti heterozygote, which is further evidence for the hypothesis that dark expresses itself only in non-agouti heterozygotes.

It should be noted at this stage that if ALL non-agouti heterozygotes are dark, then there is no evidence for the segregation of a single dark gene; as compared with the possibility of a collection of dark "polygenes" assembled in the population under study. Proof of the segregation of a single dark gene would lie in observing expected Mendelian ratios of dark:light amongst the non-agouti heterozygotes born to prepared matings.

Three points arose then, from the results of the preliminary matings. They were:

(i) An apparent segregation of dark and light agouti coat colours was taking place in the mice lines under study.

(ii) Four matings had given ratios of dark:light offspring, which did not differ from the expectation of a 1:1 ratio.

(iii) This segregation may have been the result of the segregation of homozygous (A/A) and heterozygous (A/a) agouti genotypes in the presence of one or more darkening factors.

Consequently, as a basis for further investigation, the following more specific questions were posed.

(i) Is the segregation of dark and light mice within a litter real, and not just visual separation of a continuous series?

(ii) If real, is the segregation of dark and light the result of the segregation of a single dark gene?

(iii) If a single dark gene, does this gene act as dominant or recessive to its wild-type allele?

(iv) Are all dark mice heterozygous for non-agouti?

(v) What are the effects of gene interaction as far as the agouti, dark, and dominant white loci are concerned?

The answer to these questions required a method of objectively

measuring the dark effect, which was practicable for the number of mice likely to be required to test genetic hypotheses.

GENETIC STUDIES

I. Experimental Techniques

A. Available techniques and tests of practicability.

The visible colour of an agouti mouse coat will be dependant on the following factors, (Dry 1926, Russell F.S. 1946).

(a) The lengths of the yellow bands in the respective hair types.

(b) Proportions in the coat of the three main hair types.

(c) Pigment attributes within the hairs of any one type.

Dry (1926) has investigated (a) and (b) above in the agouti mouse coat, using histological methods. The pigment attributes within the hairs have been investigated by the means of several different techniques.

(i) Visual or Photometric comparisons on prepared skins (Barrows 1934, Mather and North 1940, ~~Wright~~ WOLFF 1955). Barrows (1934) and Mather and North (1940) have reported that slight differences in mice coat colour are more easily discernible from prepared skins than from the living animal. Consequently it was decided to skin each mouse of a litter in which dark and light were supposedly segregating, and visually compare the intensity of darkness of these skins with the object of (i) obtaining a more perfect classification of dark and light, and (ii) obtaining representative dark and light skins with which other skins might be compared.

The mice used were skinned at weaning and the skins were laid underside down on blotting paper soaked in 10% formalin solution (Flux 1960) and a flat weight placed on top.

A sensitive photometric apparatus such as that used by ~~Wright~~ WOLFF (1955) when studying guinea-pig skins was not available.

Prepared skin studies were disappointing in that although the

technique gave excellent skins, comparisons among them gave no more information than classification of the live animal. The classification of any skin agreed in all cases with that of the mouse before skinning, even to the extent that "doubtful" decisions in vivo were "doubtful" decisions on the skins. Consequently there appeared to be no advantage in skinning the mice, especially as this prevented further breeding after classification. The study of skins was thus abandoned.

(ii) Colourimetric and spectrophotometric determinations on melanin solutions (Baker and Andrews 1944, Daniel 1938, Russell, E.S. 1939, Hiedenthol 1940). This technique has been confined to the study of yellow melanin which appears to be alkali soluble. However, there is some doubt (Russell L.B. and Russell W.L. 1948) as to whether true solutions are formed, and the technique was consequently not used.

(iii) Gravimetric estimation of the weight of melanin per unit weight of hair (Einesele 1937, Dunn and Einesele 1938). These authors hydrolyzed mouse hair with .6N HCl and separated the melanin by centriguging and washing. The technique was rendered less accurate by the fact that a proportion of the hair keratin does not go into solution. This proportion was estimated using hair from albino mice, and all melanin weights obtained from coloured mice were corrected by this factor. Differences in melanin weight were indicated between major colour types but not between close colour differences. It was therefore doubtful whether this technique would be sufficiently sensitive for investigating the dark gene, and consequently it was not used.

(iv) Histological examination of the hairs (Russell E.S. 1946, Grobman and Charles 1947). Russell, E.S. (1946) using the technique mentioned in the review of literature, was able to detect the effects on hair pigment of

alleles at the chinchilla, agouti, pink-eye, dilute and brown loci. To test whether the effect of the dark gene was on one or more of the seven pigment attributes this author studied, a slight modification of Russell's (1946) technique was adopted as follows.

From the plucked hair sample (described in next section), a small tuft of hairs was taken and soaked in an alcohol-xylol mixture for 4 days. These hairs were then mounted en masse on a glass slide in pure egg-albumin. The cross-sections were obtained by placing another tuft of hair from the plucked sample in a hand-microtome, from which sections were cut with a razor blade after hardening the hair with a drop of celluloid-chloroform solution. The resulting cross-sections were again mounted in pure egg-albumin. Both whole mounts and cross-sections were studied under a binocular microscope, using an oil emersion objective lens, with the condenser lens also oiled to the slide. A light green photographic filter was placed in the filter holder in an endeavour to reduce the wave-length of light being utilized. The microscope was used at a theoretical magnification of X2100, but actual magnification obtained was probably X1400 +.

(a) Whole Mounts

The three pigment attributes measured by Russell, E.S. (1946) on whole mounts were also studied here, again at successive 10 microscope field intervals down the hair starting from the 4th field. In addition, at each field studied, the diameter of the hair was recorded with the use of an ocular micrometer, which on calibration by a glass-slide micrometer at the magnification used, showed divisions of .98 μ. Both hair length and length of the sub-terminal yellow band were measured for each hair studied, both of these variables being measured in units of "microscope field diameters".

The whole mounts were disappointing in that the pigment granules were so densely crowded in the medullary cells that accurate counting of the number of granules in a cell was impossible. This discrepancy with Russell's (1946) results was confirmed by two independant observers. Cortical granules were more easily counted but this, too, became impossible in densely pigmented regions near the tip of the hair.

(b) Cross-sections

The four pigment attributes measured by Russell (1946) from cross-sections were also measured here.

The cross-sections cut by the hand-microtome were far too thick when viewed under the microscope. An attempt was thus made to cut the cross-sections on a mechanical microtome. A small tuft of hairs was set in a high melting point wax block and good sections were obtained using the microtome at a setting of 1 μ. All sections were cut at a level of approximately 1/3rd of the distance down the hair from the tip, i.e. in the black pigment zone. Again, as in the whole mounts, the pigment granules were too densely crowded to allow measurement of granule diameter. The shape of the granules could not be discovered, and the colour of the granules appeared as a fucous-brown in all cases.

The high magnification used was obviously near the limit of the microscope's resolving power. Halving the magnification of the eye-pieces greatly sharpened the image, but made the granules appear too small to be measured or counted with confidence. Consequently, attempted measurement of Russell's (1946) seven pigment attributes was discontinued in this study.

B. PROCEDURES ADOPTED

In view of the difficulties encountered in attempting to utilize Russell's (1946) technique, and the consequent decision not to use it, it became evident that the length of the agouti yellow band was the only remaining character left for study. A preliminary look at hairs from a few random dark and light mice of different ages did suggest, however, that the length of the yellow band was reduced in dark mice as compared with light mice. If this shortening of the band in dark mice was due to the dark gene, then the absence of information on pigment granule attributes was not so important. That is, the length of the yellow band could give the basis for an objective study, and this was all that was required.

As yellow band length, together with hair length and hair diameter, could all be measured at magnifications much lower than 1400 X it was decided that these three variables could be measured more efficiently on the projection microscope.

It was also decided to employ the technique used by Grobman and Charles (1947), to measure the total length of medullary black pigment per unit length of hair. The reasons for deciding to employ this relatively innacurate technique were as follows:

(i) The measurements were carried out using a projection microscope and could thus be obtained in this study with little extra time involved.

(ii) In the absence of Russell's (1946) technique, there was no other way of collecting information on black pigment.

(iii) Grobman and Charles (1947) used this method in comparing agouti-dominant white heterozygotes (A/+ Mi^{wh}/+) with agouti-wild types (A/+ +/+), both of which were genetic backgrounds for the present investigation.

(iv) The measurements obtained might be useful as a concomitant variable to the yellow band length study.

The sampling procedure is described as follows:

At weaning (21 days of age), a small tuft of hair was plucked from the sampling position with a pair of surgical forceps. One sample was plucked from each mouse, and represented an area of approximately .25 - .50 sq. cms. The sampling position was on the mid-dorsal line, slightly posterior to a point half-way between the ears and the root of the tail. This position is shown diagrammatically in Figure 7.

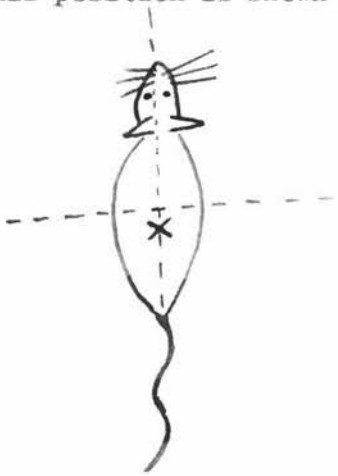


Fig. 7

Showing sampling position on the dorsal surface.

X = sampling position.

At 21 days of age all hairs of the first pelage are in the club stage, and having completed growth are easily plucked (Dry 1926, Borum 1954).

Samples were stored away from light until they were required for study.

The visual classification of each litter into grades of dark and light mice was carried out at 2 weeks of age and again at weaning. It was found that the easiest estimate of a mouse's relative degree of darkness when compared with another mouse, could be obtained by holding the two animals upside down by their tails at a distance of approximately two feet below the eye, and glancing vertically straight down the dorsal

surfaces. All comparisons were made within litters, independant of sex. The comparison within litters was necessitated by the fact that coat colour intensities alter with age (Dry 1926, Mather & North 1940), and that differences between living mice and skins from mice of the same age were difficult to assess.

In an endeavour to overcome any likely personal bias towards classifying mice into the number of groups expected from the hypothesis being tested by the mating involved, the following procedure was adopted. Two mice picked at random from the litter were compared visually as described above. The results of this comparison could be either of the following:

(i) Both mice the same

(ii) One mouse darker

In each case one mouse was placed in the appropriate of three boxes marked "Light", "Dark" and "Same". The remaining mouse was then compared with another mouse picked at random from the litter and the process repeated. When the whole litter was divided into "Light", "Dark" and "Same", the mice in the "Same" box were compared again with any one mouse from the dark group and were consequently classified as "Dark" or "Light". This process resulted in the litter being classified into two groups ("Dark" and "Light") or one group only ("Same"). If sufficient mice were available the procedure was then repeated on mice within "Dark" and "Light" groups.

All mice were given a classification, and a doubtful decision was recorded as such. This classification was carried out at 2 weeks of age when comparisons were more easily made. All mice were ear-marked and their classification recorded. At weaning the litter was again classified and the results compared with those obtained from the 2 weeks' study. Different classifications of the same mice between the two sets of

data were recorded as "doubtful" and the classification given to such mice was that of the 2 weeks' study.

Sexes, within a litter, were not classified separately as this made the mice numbers available for comparison too few. In matings where the Dominant-white gene was segregating, White heterozygotes and their normal genotypes were classified separately.

The procedure may be summarized as follows:

For each mating, the male and female parents were housed together in a cage continuously. The date the mating was made up was recorded, together with information on the origin of the two parents. Each cage was inspected daily for the appearance of litters, the date of birth and size of the litter being recorded.

At two weeks of age, litters were classified into the dark and light grades as previously described, each mouse was ear-marked, and its classification recorded. Weaning took place at 3 weeks of age. At weaning, the mice were classified again, and body weights to the nearest .2 grms were recorded. Sampling then took place. The mice were then killed or stored in cages of separate sexes, according to whether they were required for future matings or not.

From the plucked sample a small tuft of hairs was taken and spread evenly over a glass slide. A drop of pure egg-albumin was spread over a large coverslip and this was placed over the hairs on the slide. It was found that mounting the hairs directly in egg-albumin gave preparations free of air bubbles inside the hair within one hour, and consequently the alcohol-xylol treatment was not necessary.

The whole mounts were then placed in the projection microscope, using a magnification of X 500. Eight zigzag hairs were measured for each slide. These eight zigzag hairs were sampled at random from the

slide by searching for hair bulbs. Having located a bulb, the hair was followed up to the tip where measurements began. The length of solid cortex at the tip of the hair, the distal length of black medullary pigment, the length of the yellow band, and the distance from the basal termination of the yellow band to the first constriction of the hair, were all measured to the nearest .5 cms. The number of medullary cells in the distal length of black medullary pigment, and the number of medullary cells in the yellow band were also recorded. The diameter of the hair across the middle of the yellow band was measured to the nearest .5 mm.

At a position exactly half-way between the two distal constrictions, a pencil drawing, over the image, was made for a 5 cm. strip of the hair. This drawing showed the outer edges of the hair and the outlines of the medullary pigment clumps. The 5 cm. strip was always begun against the outer edge of the first medullary clump to be included in the drawing.

At the completion of the drawing, the microscope was turned over to X 86 magnification, and the total length of the hair was measured. The total length was always estimated from the sum of lengths of the sections between constrictions, all measured to the nearest .5 cms. The proximal section was measured in two parts, which were: the distance from the proximal constriction to the point where melanin clumps ceased to be evident, and from this point to the base of the bulb. The slide was then searched for another bulb, the magnification turned back to X 500, and the process repeated, until eight hairs had been measured.

The 5 cm. long projection drawings were measured later. The mean of three diameters across the hair to the nearest .5 mm. was measured and the total length of medullary pigment along the longitudinal mid-axis was also estimated, again to the nearest .5 mm.

Samples were taken from the first pelage only, as mice were classified visually on the appearance of the first coat. Only zigzag hairs were studied as these represent about 80% of the dorsal coat.

C. SAMPLING NUMBERS

In order to derive some idea of the sample size required ~~in order~~ to discriminate between dark and light mice on the basis of yellow band length, a preliminary analysis of variance was carried out. As it was necessary to first have a reliable visual classification to place mice in dark and light groups, the first 25 $Mi^{wh}/+$ mice available from matings Mi 1 to Mi 5, were used for this analysis.

A similar analysis was also carried out on the estimates of medullary pigment length per 5 cm. length of hair. The estimates of yellow band length and black pigment used for each mouse were the mean value for 8 hairs.

(i) The Statistical Model

The analysis of variance model was

$$y_{ijkl} = \mu + l_i + s_j + c_k + e_{ijkl}$$

$i = 1.....7$

$j = 1, 2$

$k = 1, 2$

$l = 0, 1, 2$

where

y_{ijkl} is the yellow band length (or black pigment length) of the l^{th} mouse, in the ijk^{th} cell.

μ is a constant effect common to all mice in the sample

l_i is an effect common to all mice from the i^{th} litter.

s_j is an effect common to all mice of the j^{th} sex, where s_1 = males, and s_2 = females.

c_k is an effect common to all mice of the k^{th} colour group where c_1 = dark, and c_2 = light.

e_{ijkl} is an error or residual peculiar to each observation.

The l_i, s_j, c_k and μ are considered to be unknown fixed constants.

The e_{ijkl} are assumed to be normally and independantly distributed with the same variance σ^2 around a mean of zero.

For the purposes of the preliminary analysis, matings were combined.

The model was analysed by the method of fitting constants by least squares (Kempthorne 1952). The estimates so obtained are minimum variance unbiased.

The original least squares equations are shown in Table 12.

Table 12

The least squares equations for the preliminary analysis of yellow band length and black pigment length.

	Mean	Litters							Males	Females	Dark	Light	Yellow Band length (cms)	Black Pigment length (mms)
	μ	l_1	l_2	l_3	l_4	l_5	l_6	l_7	s_1	s_2	c_1	c_2	Σy	Σy
μ	25	4	2	4	3	2	7	3	10	15	12	13	780.687500	532.990000
l_1	4	4							1	3	2	2	101.125000	91.310000
l_2	2		2						2	0	1	1	67.812500	39.880000
l_3	4			4					2	2	2	2	123.750000	80.120000
l_4	3				3				1	2	1	2	95.375000	61.250000
l_5	2					2			0	2	1	1	62.250000	41.190000
l_6	7						7		3	4	4	3	239.187500	154.740000
l_7	3							3	1	2	1	2	91.187500	64.500000
s_1	10	1	2	2	1	0	3	1	10	0	6	4	309.750000	216.500000
s_2	15	3	0	2	2	2	4	2	0	15	6	9	470.937500	316.490000
c_1	12	2	1	2	1	1	4	1	6	6	12	0	346.250000	265.250000
c_2	13	2	1	2	2	1	3	2	4	9	0	13	434.437500	267.740000

In order to solve the least squares equations, the l equations were solved for $(\mu + l_i)$ in terms of the s's and the c's. These expressions were then substituted in the s and c equations to give equations only in the s's and c's. These four reduced equations have no unique solution since the rank of the matrix of coefficients is only 2. To solve the system, the non-estimable conditions $\sum s_j = 0$ and $\sum c_k = 0$, were imposed. Under this type of restriction the estimate of μ is the mean of a hypothetical population in which all sub-class numbers are equal. The resulting two equations in s_1 and c_1 were solved by use of the Grout method for solving simultaneous equations (Titner 1959). As there were only two sex classifications and two colour classifications, $s_2 = - s_1$, and $c_2 = - c_1$, these estimates were then substituted back in the original equations which were then solved for the $(\mu + l_i)$. From these estimates, the reduction in sums of squares due to the fitting of constants was obtained.

The adjusted mean squares for effects were each tested against the mean square for error + interaction with the appropriate degrees of freedom, if the mean squares for error and interaction were of the same magnitude (Rao 1952).

The sums of squares due to all interactions were estimated as the difference

Between sub-class ss - R (μ, l, s, c)

(Kempthorne 1952); and were tested against the within sub-class sums of squares or error. The interactions must be shown to be non-significant for the assumption of additivity implicit in the model to hold. In the event of the additivity assumption not holding, the analysis of variance must be carried out within the interacting groups (Kempthorne 1952, Rao 1952).

(ii) RESULTS

(a) Yellow Band Length

The analysis of variance to test interactions is shown in Table 13. As interaction was not significant, the analysis was continued on the assumption that the sub-classification effects were additive.

Table 13

Analysis of Variance of interaction (Yellow Band Length)

Source		d.f.	s.s.	M.s.	F	P
Total	A	25	24866.99			
R (μl,s,c)	B	9	24752.43			
Between sub-classes	C	20	24824.66			
Interaction	C-B	11	72.23	6.57	.77	N.S.
Error	A-C	5	42.33	8.47		

F Value 11 & 5 d.f. p = .05 is 4.7

Estimation of the effects and the resulting sums of squares gave the analysis of variance shown in Table 14. There were significant effects associated with litters and colours.

Table 14

Analysis of variance of effects (Yellow band length)

Source		d.f.	s.s.	M.s.	F.	P
Total	A	25	24866.99			
$R(\mu, l, s, c)$	B	9	24752.43			
Error & Interaction	A-B	16	114.56	7.16		
$R(\mu, l, s)$	C	8	24619.07			
Colour Group	B-C	1	133.36	133.36	18.62	**
$R(\mu, l, c)$	D	8	24748.17			
Sex	B-D	1	4.26	4.26	.59	N.S.
$R(\mu, s, c)$	E	3	24509.87			
Litters	B-E	6	242.56	40.43	5.65	**

F values 1 and 16 d.f. P = .01 is 8.68
P = .05 is 4.54

6 and 16 d.f. P = .01 is 4.32
P = .05 is 2.79

(b) Black Pigment

The analysis of variance of interaction is shown in Table 15.

Table 15

Analysis of variance of interaction (Black pigment length)

Source		d.f.	s.s.	M.s.	F.	P
Total	A	25	11446.9			
R (μ,l,s,c)	B	9	11406.4			
Between sub-classes	C	20	11433.9			
Interaction	C-B	11	27.5	2.5	1.0	N.S
Error	A-C	5	13.0	2.6		

F Value 11 and 5 d.f. P = .05 is 4.7

The analysis of variance of effects is shown in Table 16.

Table 16

Analysis of variance of effects (Black pigment lengths)

Source		d.f.	s.s.	M.s.	F	P
Total	A	25	11446.9			
R (μ,l,s,c)	B	9	11406.4			
Error & Interaction	A-B	16	40.5	2.5		
R (μ,l,s)	C	8	11396.9			
Colour groups	B-C	1	9.5	9.5	3.8	.05-.10
R (μ,l,c)	D	8	11402.9			
Sex	B-D	1	3.5	3.5	1.4	N.S
R (μ,s,c)	E	3	11377.6			
Litters	B-E	6	28.8	4.8	1.9	N.S

F Values .. See Table ~~20~~ 14

(iii) Discussions

(a) Yellow Band Length

The pertinant results from the preliminary analyses were:

(i) Statistically significant differences at the 1% level existed between the mean yellow band lengths of the dark and light mice groups. Consequently it may be supposed that the dark gene/genes had an effect of reducing the length of the yellow agouti band in zigzag hairs from the position sampled. Also, with the sampling numbers used in this analysis (8 hairs per mouse, and approximately 12 to 13 mice in each group), the technique was sufficiently accurate to detect such differences. It should be noted that these results apply only to differences in yellow band length between mice of the genotypes $Mi^{wh}/+d/+Aa$ and $Mi^{wh}/+,+/+,Aa$.

(ii) There were statistically significant differences at the 1% level between litter means. This suggested the necessity of either correcting for litter effects, or carrying out future analyses on a within litter basis.

(iii) There was no evidence of sex effects.

As the numbers of mice used in this analysis did not permit the differences between parent groups to be estimated, effects common to specific parent pairs (such as genetic background effects and natural effects) are completely confounded with specific litter effects (such as litter size and mean litter weaning weight).

(b) Black Pigment Length

There was no statistically significant difference between sexes or between litters for the black pigment lengths of mice hairs as measured by the method of Grobman and Charles (1947). However, the differences between dark and light groups had a 7% probability of arising due to

chances in sampling. This suggested that, with larger mice numbers, and/or comparing genotypes with larger differences in their dark phenotypes, the technique would be worth persevering with.

With a minimum of 12 - 13 mice in each group, the techniques appear satisfactory for detecting differences between dark and light mice groups, on at least one objective basis, and for at least one genotypic difference.

II. GENETIC RESULTS BASED ON VISUAL SEGREGATIONS

A. Introduction.

The matings to be described were designed to assist in answering the questions posed by the results of the preliminary matings. The preliminary matings results indicated that the dark phenotype was genetically determined. Consequently, the nature of the genetic situation (single recessive, single dominant, or polygenic) causing the dark phenotype, and the nature of any interaction at the dark and agouti loci, was investigated as follows.

The first series of matings (d_9 to d_{17}, D_1 to D_4, B.1 and B.2) were planned with the object of investigating the segregation of dark on a homozygous agouti (A/A) background. These matings were also expected to give information on the dominance relations of the d and d^+ alleles.

The second series of matings (Mi.1 to Mi.5) were designed to test the hypothesis that interaction occurs between the dark and agouti loci, by attempting to obtain the segretation of dark on heterozygous agouti (A^+/a, A^+/a^t, A^w/a, A^w/a^t) backgrounds. Again, information was expected on the dominance relations of the d and d^+ alleles.

The third section which consisted of one mating (MiT), was designed as an attempt to relate the results of the previous two sections on a within litter basis. That was, investigate the phenotypes of dark and light mice on both A^w/A^w and A^w/a^t backgrounds.

The last mating (U.1) was designed to test the conclusions drawn from the MiT mating.

In view of the difficulty involved in classifying mice as dark or light by eye, the Dominant White allele (Mi^{wh}) was introduced in the heterozygous state in matings Mi.1 to Mi.5 and Mi.T, in the hope

that the dark phenotype might show more obviously on the lighter coat of $Mi^{wh}/+$ mice.

In most cases, the designs of the above matings, and the conclusions drawn from their results, were partially dependant on the results obtained from the preceding matings. Consequently, each of these four series of matings is described in the above order to facilitate presentation.

B. The Matings and Visual Results

1. Homozygous Agoutis

In an attempt to obtain any dark mice that might be homozygous for agouti (A/A) the following nine matings were made up.

d_9, d_{11}, d_{12}, d_{13}, d_{14}. dark ♂ x c_{57} ♀ (a/a) black

d_{10}, d_{15}, d_{16}, d_{17}. dark ♀ x c_{57} ♂ (a/a) black

The dark parents of these matings were stored offspring from the preliminary matings, and were clearly of dark phenotype. The results are shown in Table 17.

Table 17

The numbers of offspring from matings d_9 to d_{17} classified according to sex, dark and agouti.

Mating	Males			Females			Total
	Dark	Light	Non-agouti	Dark	Light	Non-Agouti	
d_9	0	0	4	2	0	2	8
d_{10}	1	0	1	1	0	3	6
d_{11}	2	0	1	0	0	1	4
d_{13}	2	0	3	4	0	1	10
d_{14}	1	0	2	2	0	0	5
d_{16}	3	0	2	2	0	2	9
d_{17}	1	0	1	3	0	2	7
Total	10	0	14	14	0	11	49

Table 17 (cont)

Mating	Males			Females			Total
	Dark	Light	Non-Agouti	Dark	Light	Non-Agouti	
d_{12}	4	0	0	5	0	0	9
d_{15}	4	0	0	4	0	0	8
Total	8	0	0	9	0	0	17

The dark ♂ parent of d_{12} and the dark ♂ parent of d_{15} became the basis of an inbred line based on brother-sister mating in each generation. These two mice were presumed to be agouti (A^W/A^W) homozygotes ($p < .01$ of being accepted as agouti homozygotes when really non-agouti heterozygotes, in both cases). The first four inbred matings were designated D_1, D_2, D_3 and D_4, where the subscript refers to the generation.

From matings D1 to D4, a total of 37 mice were bred, all of which were yellow-bellied (A^W/A^W) agouti in phenotype, and all appeared dark in phenotype. The darkness of all mice in matings D1 to D4 did not appear to be as intense as that of the dark parents used in matings d_9, d_{10}, d_{11}, d_{13}, d_{14}, d_{16} and d_{17}, which were non-agouti heterozygotes.

In an attempt to obtain the segregation of dark and light on a homozygous agouti background, offspring of matings D2 and D3 were outcrossed to the C B A inbred line which is homozygous for grey-bellied agouti (A^+/A^+) and in which dark mice have not been observed. Three of these matings were established as follows:

B.1 C B A ♂ (A^+/A^+) (light) x D.2 & D.3 ♂ (A^W/A^W) (dark)

↓

54 offspring (A^W/A^+) all light

From the offspring of mating B.1, three males were backcrossed

to their respective dark mothers as follows

B.2 B.1 ♂ (A^W/A^+) light x D.2 & D.3 ♂ (A^W/A^W) dark

↓

37 offspring (A^W/A^+ & A^W/A^W)

The results of mating B.2 are shown in Table 18.

Table 18

Males			Females			Total
Dark	Light	Unknown	Dark	Light	Unknown	
4	3	8	3	5	14	37

Matings d_{12} and d_{15} indicate that at least one dark genotype may express itself in the absence of the a/+ genotype. This does not preclude the possibility that the genotype at the agouti locus might influence the phenotype expression of some dark genotypes. This possibility is quite likely in view of the two dark parents of matings d_{12} and d_{15} appearing slightly less dark than the dark parents of matings d_9, d_{10}, d_{11}, d_{13}, d_{14}, d_{16} and d_{17}, all of which are shown in Table 17 to be of the a/+ genotype.

The series of inbred matings D.1 to D.4 which produced only dark mice in each generation, would suggest that such mice were homozygous for the dark gene/genes, if the dark gene/genes are assumed to be recessive to their wild-type alleles. Evidence in sup ort of the assumption that the dark genes are recessive is obtained from mating B.1, in which all offspring were of light phenotype. The possibility that the dark genes might be dominant but do not express themselves on the A^W/A^+ background of the B.1 offspring, has been rejected on the basis that dark mice have been observed in the original body weight - tail length lines on A^+/A^+

backgrounds (Cockrem 1962).

Evidence then would suggest that the dark gene/genes are recessive and homozygous in all mice in matings D.1 to D.4. On the assumptions that the dark phenotype is due to a single pair of alleles, and that these alleles are not present in the c_{57} line, the genotypes of mice involved in matings d_{12}, d_{15}, D.1, D.2, D.3, and D.4, would be as follows:

d_{12} and d_{15} $\quad ddA^{W}A^{W} \times ++aa$

$\downarrow$

$d+A^{W}a$

D.1 to D.4 $\quad ddA^{W}A^{W} \times ddA^{W}A^{W}$

$\downarrow$

$ddA^{W}A^{W}$

As offspring of these two sets of matings were all graded as dark, but under the above hypothesis have different genotypes, it became of interest to compare the intensity of darkness of the two sets of offspring. Consequently the male parent of mating D.2 was also mated to a c_{57} female. The offspring of this mating which were all dark were then compared with the offspring of mating D2 at slightly different ages. As near as could be judged by eye, the D.2 offspring were the darker. It was not thought that the difference in intensity would be evident to the observer unless the difference was being especially looked for.

With the same two assumptions holding, the genotypes of mice involved in matings B.1 and B.2 are :

B.1 C B A ♂ $(++A^{+}A^{+})$ x D.2 and D.3 ♂ $(ddA^{W}A^{W})$

↓

$d+A^{W}A^{+}$

B.2 B.1 ♂ $(d+A^{W}A^{+})$ x D.2 and D.3 ♂ $(ddA^{W}A^{W})$

↓

$ddA^{W}A^{W}$ } DARK
$ddA^{W}A^{+}$ }
$d+A^{W}A^{W}$ } LIGHT
$d+A^{W}A^{+}$ }

As all 54 offspring of mating B.1 were yellow-bellied in phenotype, it is evident that the dark D.2 and D.3 parents were homozygous A^{W}/A^{W} ($p < .001$ of being accepted as A^{W}/A^{W} when really A^{W}/A^{+})

The offspring of mating B.1 were clearly of light phenotype, with not the slightest trace of duskiness evident on the dorsal surfaces. Consequently the relationship of genotype to intensity of darkness would appear to be:

Genotype $ddA^{W}A^{W} > d+A^{W}a > d+A^{W}A^{+}$

Phenotype dark ⟶ less dark ⟶ light

These relationships have been based entirely on evidence from separate matings; that is, no information on within litter segregation has been utilised so far. According to hypothesis, mating B.2 should segregate dark and light offspring in a 1 : 1 ratio. Table 18 indicates that on a within litter basis this segregation was very difficult to detect, if the segregation indeed exists. Such a poor visible segregation might be explained in the following ways:

(i) The assumption that the dark phenotype is due to a single pair of alleles may not be valid. If the dark phenotype is due to a series of recessive polygenes assembled in the D.1 to D.4 inbred line, then results such as those obtained from mating B.2 are not unexpected.

(ii) The expression of the $ddA^{W}A^{+}$ and $ddA^{W}A^{W}$ genotypes may be so influenced by other genetic and environmental factors that considerable overlapping of phenotypic values of the d+ and dd genotypes occurs.

(iii) The segregation of dd and d+ may be real and non-overlapping in phenotypic expression but the difference cannot be detected with the naked eye.

As 15 of the 37 mice from mating B.2 were visibly classified into dark and light groups, it is likely that some genetic segregation took place. However, the results cannot be accepted as representing a 1 : 1 ratio.

2. HETEROZYGOUS AGOUTIS

To test the hypothesis that mice of the genotype d+Aa have a dark phenotype, matings Mi.1, Mi.3, Mi.4 and Mi.5 were investigated. The C Y X inbred line was chosen for the outcross as it had been maintained for the segretation of the dominant white heterozygote, and it was hoped that the dark genotype might express itself more clearly on the lighter phenotypic background of the $Mi^{wh}/+$ genotype. The C Y X parents of matings Mi.1, Mi.4 and Mi.5 were homozygous for black-and-tan (a^{t}/a^{t}). The C Y X parent of mating Mi.2 was a non-agouti homozygote (a/a). It was thus hoped to compare any segregation of d/+ and +/+ on both A/a^{t} and A/a backgrounds; that is, compare the efficiency of the a^{t} and a alleles as modifiers of dominance at the dark locus/loci. Unfortunately mating Mi.2 failed to breed. However, this mating was not repeated as

dark and light Dominant white heterozygotes were being observed among litters of matings Mi.1, Mi.4 and Mi.5, and the C Y X parent of mating Mi.3 which was originally thought to be of the a^t/a^t genotype was shown to be a^t/a by the appearance of some grey-bellied mice amongst his offspring.

In establishing these matings, one male and three females from the B.1 mating were outcrossed to the C Y X inbred line as follows:-

Mi.1 B.1 ♂ $(A^w/A^+ +/+)$ x CYX ♂ $(a^t/a^t Mi^{wh}/+)$

Mi.3 CYX ♂ $(a^t/a Mi^{wh}/+)$ x B.1 ♂ $(A^w/A^+ +/+)$

Mi.4 CYX ♂ $(a^t/a^t Mi^{wh}/+)$ x B.1 ♂ $(A^w/A^+ +/+)$

Mi.5 CYX ♂ $(a^t/a^t Mi^{wh}/+)$ x B.1 ♂ $(A^w/A^+ +/+)$

The results from these four matings are shown in Tables 19 and 20.

Table 19

Numbers of Dominant White heterozygotes from matings Mi.1, Mi.3, Mi.4 and Mi.5 classified according to litter, sex & dark.

Mating	Litter	Males		Females		Total
		Dark	Light	Dark	Light	
Mi.1	1	1	0	1	2	4
	2	1	1	0	0	2
Total		2	1	1	2	6
Mi.3	1	1	1	1	1	4
	2	1	0	0	2	3
	3	0	0	1	1	2
	4	2	1	2	2	7
	5	1	1	2	2	6
Total		5	3	6	8	22
Mi.4	1	1	1	1	1	4
	2	1	1	0	1	3
Total		2	2	1	2	7
Mi.5	1	0	1	1	1	3
	2	0	0	0	2	2
	3	2	1	0	2	5
Total		2	2	1	5	10
Total	12	11	8	9	17	45

Table 20

Numbers of wild-type Agouti phenotypes from matings Mi.1, Mi.3, Mi.4 and Mi.5, classified according to sex and dark.

Mating	Males			Females			Total
	Dark	Light	Unknown	Dark	Light	Unknown	
Mi.1	0	0	4	0	0	0	4
Mi.3	1	2	10	1	3	6	23
Mi.4	0	0	2	1	0	7	10
Mi.5	1	0	3	2	2	7	15
Total	2	2	19	4	5	20	52

All 97 offspring from matings Mi.1, Mi.3, Mi.4 and Mi.5 were outcrossed to mice of the c_{57} inbred line, in an attempt to classify the genotypes at the agouti locus for each mouse. Mice of the genotype A^{+}/a whose phenotype can be distinguished from the phenotype of A^{w}/a^{t}, A^{w}/a, and A^{+}/a^{t} genotypes, were not included in such crosses.

The offspring of mating B.1 were expected to be dark heterozygotes ($d+A^{w}A^{w}$) so that the genotypes of mice involved in matings Mi.1 to Mi.5 were :

Mi.1 - 5. $d+,A^{w}A^{+},++ \quad x \quad ++,a^{t}a^{t},Mi^{wh}+$

$d+,Aa^{t},Mi^{wh}+$
$d+,Aa^{t},++$
$++,Aa^{t},Mi^{wh}+$
$++,Aa^{t},++$

The dominant allele at the agouti locus of all offspring may

be either A^w or A^+, while the recessive allele at the agouti locus of offspring from Mi.3 may be either a^t or a.

Consequently, if the genotype d+Aa shows a dark phenotype, both dark and light mice are expected in equal numbers from the offspring of these matings.

On the Dominant white background ($Mi^{wh}/+$), the segregation of dark and light mice was clearly observed in all litters.

As the ultimate sub-class members of Dominant white mice from matings Mi.1, Mi.3, Mi.4 and Mi.5, were less than 5, a chi-square analysis was carried out on the totals, with matings and litters combined. The chi-square analysis, testing for deviation from expected ratios of 1 : 1 for sex, dark and linkage of sex and dark, is shown in Table 21. There was no evidence of deviation from expectation from any scource.

Table 21.

χ^2 estimates for the data in the total line of Table 19.

Source	χ^2	df	p
Male vs Female	.56	1	.30 - .50
Dark vs Light	1.09	1	.20 - .30
Linkage	2.69	1	.10 - .20
Total	4.34	3	.20 - .30

By test-mating all offspring to c_{57} (a/a) mice, it was found that the genotypes A^w/a, A^+/a, A^w/a^t, and A^+/a^t, were all represented in both dark and light mice. Hence the segregation of these four alleles among the offspring was not responsible for the dark and light segregations. To test the possibility that the dark factor may have

resided within the C.Y.X. inbred line, the ♂ parent of mating Mi.3 was out-crossed to a female from the C.B.A. inbred line as follows:

CYX (Mi^{wh}/+ a^{t}/a) x CBA (++$A^{+}A^{+}$)

All 19 offspring from this mating were of light phenotype, regardless of the alleles they carried at the agouti and Dominant White loci. It is unlikely then, that the dark factor was in the CYX line, and so must have entered in the heterozygous state (d+) with the wild-type agouti parents which were offspring of mating B.1. As these wild-type agouti parents were shown to be of light phenotype, the results shown in Table 19 present good evidence for the recessive nature of the dark gene. It was concluded from these results obtained on the Dominant White heterozygotes, that:

(i) A true segregation of dark and light mice took place on Mi^{wh}/+,A/a backgrounds in a ratio not deviating significantly from 1:1.

(ii) This segregation was due to a single recessive gene (d/+); so that d+aA mice were dark and ++Aa mice were light.

(iii) Consequently, mutual reduction of dominance was occurring at the dark and agouti loci.

(iv) All four genotypes A^{w}/a, A^{+}/a, A^{w}/a^{t} and A^{+}/a^{t}, were successful in modifying the dominance relations at the dark locus.

The results obtained from the wild-type agouti offspring of matings Mi.1 to Mi.5, shown in Table 20, do not however support the above conclusions. The segregation amongst these mice was very difficult to identify, only 13 mice being clearly classified into dark and light groups, with 39 mice classified "unknown". All that can be said regarding the latter group is that they were neither clearly dark nor light, and differences in coat colour intensity between them could not be

confidently discerned. However, as 6 dark and 7 light mice were identified, and the segregation was clearly apparent on the Dominant White agoutis, it was concluded that the same segregation was taking place, but overlapping or near-overlapping dark and light phenotypes made reliable classification extremely difficult by eye. The results of yellow band length studies to be discussed later, suggest at least one reason why the segregation should be easier to detect by eye in Dominant White rather than wild-type agouti mice.

3. Homozygous and Heterozygous Agoutis

In an attempt to observe the segregation of dark homo- and heterozygotes on both A^wA^w and A^wa^t backgrounds, a dark Dominant White female ($d/+,A^w/a^t$, $Mi^{wh}/+$) from mating Mi.3, was mated to a dark male (d/d? A^w/A^w) from mating D.4 of the inbred line which was supposedly homozygous for dark. The genotypes of mice involved in this mating were expected to be as follows:

Mi.T D.4 ♂ ($d/d,A^w/A^w,+/+$) x Mi.3 ♂ ($d/+,A^w/a^t,Mi^{wh}/+$)

$d+A^wa^t$	On both $Mi^{wh}/+$ and $+/+$ backgrounds
ddA^wa^t	
$d+A^wA^w$	
ddA^wA^w	

The results are shown in Table 22.

Table 22

Numbers of mice from mating Mi.T classified according to Dominant White and Dark. Sexes have been combined.

Litter	Dominant White Agouti			Wild-Type Agouti			Total
	Very Dark	Dark	Light	Very dark	Dark	Light	
1	0	4	2	0	5	2	13
2	0	6	1	0	3	1	11
3	1	5	1	1	1	1	10
4	0	4	0	0	2	1	7
5	1	1	0	4	0	1	7
Total	2	20	4	5	11	6	48
A^wA^w	0	8	3	2	5	6	24
A^wa^t	2	12	1	3	6	0	24

Only three phenotypes were discernable. On both $Mi^{wh}/+$ and $+/+$ backgrounds, the mice were divisable into a total of 38 darks and 10 lights. The 38 dark mice were divided into 7 "very darks" and 31 darks. Consequently it appeared as if two of the expected genotypes were being graded together. The results of a Chi-square analysis to test for deviations from expected 1:2:1 ratios for the Dominant white agoutis, wild-type agoutis, and the combined data is shown in Table 23.

Table 23

χ^2 estimates for testing deviations from an expected 1:2:1 ratio for the data in the total line of Table 21.

Source	χ^2	df	p
Dom. White Agouti	7.79	2	.02 - .05
Wild-type Agouti	.10	2	.95 - .98
Total	7.89	4	.05 - .10
Combined Data	4.15	2	.10 - .20
Heterogeniety	3.74	2	.10 - .20

The ratio of 2 very dark : 20 dark : 4 light obtained from the Dominant Whites, differed significantly from expectation at the 5% level. The results from the wild-type agoutis gave excellent agreement with expectation (p = .95 - .98), while the combined data was just in agreement (p = .10 - .20). As the heterogeniety item was not significant at the 5% level (p = .10 - .20), both the Dominant White and wild-type agouti groups were accepted as being in agreement with each other, and showing agreement with the combined data. However, there is little doubt that there were a large number of mis-classifications among the Dominant white offspring. The heterogeniety item's low level of significance may reflect "poor classification" as has been suggested by Mather (1938)

Table 22 also shows the numbers of A^wA^w and A^wa^t genotypes occurring in each of the three phenotypic groups, found by test-mating all offspring to c_{57} black (a/a) mice. Of the 10 mice graded as light, 9 were A^wA^w, while only 1 was A^wa^t. Of the 7 mice graded as very dark, 5 were A^wa^t, while only 2 were A^wA^w. If, as is very likely, the 1 light A^wa^t mouse

and the two very dark A^wA^w mice represent mis-classifications, then the genotypes of the three groups could be

ddA^wa^t - very dark

ddA^wA^w
$d+A^wa^t$ (- dark

$d+A^wA^w$ - light

The difficulty in detecting differences in dark effect between d+Aa and ddAA mice has already been discussed. The main scource of error then in classifying the Mi.T offspring has been the placement of some very dark and some light mice in the dark group.

The conclusions reached from the results of the MiT mating may be summarised as follows:

(i) A recessive dark gene was segregating among the offspring as d/d versus d/+ on both homozygous and heterozygous black-and-tan backgrounds.

(ii) The segregation did not differ significantly in the total data from an expectation of 1 very dark : 2 dark : 1 light.

(iii) A shortage of very dark and light mice, although not significant in the combined data, was observed.

(iv) This shortage was probably due to the mis-classification of some very dark and some light mice, as dark.

(v) The very dark mice have the genotype ddA^wa^t.

(vi) Mice of the genotypes ddA^wA^w and $d+A^wa^t$ have a dark phenotype and are indistinguishable by eye.

(vii) Mice of the genotype $d+A^wA^w$ have a light phenotype and are visually indistinguishable from normal non-dark mice.

(viii) The apparent mutual reduction of dominance at the dark

and agouti loci is unusual in that both homozygous (d/d) and heterozygous (d/+) dark mice reduce the dominance of the A^w allele over a^t such that A^w/a^t genotypes may be darker than A^w/A^w genotypes, while heterozygous agouti (A^w/a^t) may completely remove dominance of the d^+ allele over d.

4. THE "VERY DARK" GENOTYPE

The "very dark" wild-type agouti male from the third litter of the MiT mating was crossed to a dark female from mating D.4 as follows:

U.1 MiT ♂ (A^w/a^t +/+) x D.4 ♂ (A^w/A^w +/+)

Mating U.1 was established to test that the genotype of the very dark mice from matings Mi.1 to Mi.5 was indeed ddA^wa^t, and at the same time to observe the effect of A^wA^w versus A^wa^t in homozygous dark mice, without other segregations complicating the classifications. The expected genotypes of mice involved in this mating were as follows:

(Very dark) Mi.T ♂ (d/d,A^w/a^t) x D.4 ♂ (d/d,A^wA^w) (Dark)

↓

ddA^wa^t - very dark

ddA^wA^w - dark

The Dominant White allele Mi^{wh} was not used in this mating. The results are shown in Table 24.

Table 24

Numbers of mice from mating U.1 classified according to litter, sex and dark.

Litter	Males		Females		Total
	Dark	Very Dark	Dark	Very Dark	
1	1	1	1	2	5
2	2	1	0	1	4
3	2	0	1	2	5
4	1	3	0	2	6
Total	6	5	2	7	20
A^wA^w	5	0	2	0	7
A^wa^t	0	3	0	6	9

The segregation of very dark and dark was clearly observed. Not all offspring were test-mated to c_{57} black (a/a) mice, but from those that were, it is evident that all very dark mice were agouti heterozygotes, while all dark mice were agouti homozygotes. A ratio of 8 dark : 12 very dark does not differ significantly from the expectation of a 1:1 ratio in a sample of 20 mice ($p = .30$). The results of this mating support the previous conclusions.

C. CONCLUSIONS

The results of matings d_9 - d_{17}, D_1 - D_4, B.1, MiT, U.1 and the Dominant White heterozygotes from matings Mi.1-5, suggest good evidence for the segregation of a single recessive dark gene which has its phenotypic effect modified by A/a heterozygotes.

The genotype-phenotype relationships for the dark and agouti loci would appear to be as follows:

++,AA)
) - light or "normal"
d+,AA))

ddAA)
) - dark
d+Aa)

ddAa - very dark

The dominant agouti allele may be either A^w or A^+, while the recessive allele may be a^t or a. The same genotype-phenotype relationships hold for both $Mi^{wh}/+$ and +/+ backgrounds although the $Mi^{wh}/+$ mice are lighter.

However, these results were not confirmed by mating B.2 and the wild-type agouti offspring of matings Mi.1-5. Assumptions have been made that the absence of an expected segregation is due to either overlapping or near-overlapping phenotypic values, making visible classification difficult. Although some evidence has been presented in support of these assumptions, they cannot be considered as proved. Also, through the limitations of cage numbers, space, time, and the amount of work involved in the objective studies, numbers of parents and offspring in all matings are small. Thus, tests on observed ratios can only show non-disagreement

with the expected ratios, but cannot be interpreted as proving agreement. Consequently, conclusions based on the results of matings where segregations were observed, will not be accurate if the observed ratios are representative of ratios other than those expected.

In view of the difficulty of visual classification of genotypes whose phenotypic effects are small and perhaps over-lapping, it is probably not possible to prove genetic segregation without the aid of some accurate, non-overlapping objective measure of phenotype.

The use of the $Mi^{wh}/+$ heterozygote as a background for the dark-light segregation has greatly facilitated classifications. However, in the absence of objective measures, classification of phenotype is still not easily or accurately performed. Consequently the present results only suggest the conclusions drawn in this study, and they cannot be accepted as proved.

III. RESULTS BASED ON OBJECTIVE MEASUREMENTS OF THE DARK PHENOTYPE

A. INTRODUCTION

The problem of visually distinguishing between a possible quantitative continuous distribution of dark phenotypes, and the two discrete populations of a simple Mendelian 1:1 segregation, suggests the requirement for some objective measure of phenotype. If the objective measurement can be obtained at a physiological level nearer the original action of the gene than visual estimation permits, a more accurate classification of phenotypes may result. Even if some variation is still present in the objective measurement, overlapping phenotypic values may be reduced or eliminated entirely. In this investigation, the difficulties of discriminating between very similar phenotypes by eye, necessitated finding a means of replacing visual phenotype by an objective measurement.

Such an approach has two requirements. First, the two or more groups must be separated visually to some degree in order to obtain groups of near similar genotypes on which to look for an objective factor. Second, having found an objective measurement, the relationship of the visual phenotypic group to the measurement must be shown.

That the length of the yellow agouti band may be a satisfactory measurement of the dark effect, has been indicated by the results from the trial of techniques. Consequently this variable was utilised in these studies.

As it was first necessary to have phenotype groups as near homogeneous as possible, only results from the U.1 and Mi.T matings, and the Dominant White agoutis ($Mi^{wh}/+$) from matings Mi.1-5, were used in this study.

B. STATISTICAL ANALYSES

The analyses of variance models were all analysed by the method of fitting constants by least squares, which is described in the section on Trials of Techniques. The models used will be described in the appropriate sections.

The analyses of covariance were carried out following Federer (1957), except that the reductions in cross-products were obtained from the analyses of variance by multiplying the R.H.S. of the y (or x) normal equations by the appropriate fitted constants of x (or y). (Cockrem 1962).

C. THE DARK GENE IN RELATION TO LENGTH OF THE YELLOW AGOUTI BAND.

1. The Dominant White ($Mi^{wh}/+$) of Matings Mi.1-5

(i) Method of Analysis.

All values used in this analysis were the means of 8 hairs for each of the 45 mice used. The data were analysed by the use of the model:

$$y_{ijkl} = \mu + l_i + s_j + c_k + e_{ijkl}$$

Where $i = 1,2,.....12.$

$j = 1,2$

$k = 1,2$

$l = 0,1,2.$

The l_i represent litter effects, the s_j sex effects (s_1 = male, s_2 = female), and the c_k colour classification effects (c_1 = dark, c_2 = light). The least squares equations are shown in Table 25.

To simplify the analysis, effects due to the four sets of parents were not estimated. Thus the 12 litters represent the combined matings Mi.1, Mi.3, Mi.4, and Mi.5.

Table 25

The least squares equations for yellow band lengths from Matings Mi.1-5

μ	l_1	l_2	l_3	l_4	l_5	l_6	l_7	l_8	l_9	l_{10}	l_{11}	l_{12}	s_1	s_2	c_1	c_2	Σ_y
l_1	4												1	3	2	2	101.12
l_2		2											2	0	1	1	67.81
l_3			4										2	2	2	2	123.75
l_4				3									1	2	1	2	95.38
l_5					2								0	2	1	1	62.25
l_6						7							3	4	4	3	239.19
l_7							6						2	4	3	3	188.13
l_8								4					2	2	2	2	144.31
l_9									3				2	1	1	2	95.19
l_{10}										3			1	2	1	2	91.18
l_{11}											2		0	2	0	2	66.37
l_{12}												5	3	2	2	3	161.56
s_1	1	2	2	1	0	3	2	2	2	1	0	3	19	0	11	8	601.68
s_2	3	0	2	2	2	4	4	2	1	2	2	2	0	26	9	17	834.56
c_1	2	1	2	1	1	4	3	2	1	1	0	2	11	9	20	0	591.30
c_2	2	1	2	2	1	3	3	2	2	2	2	3	8	17	0	25	844.94

(ii) Results.

The results of the analyses of variance for both effects and interactions are shown in Table 26. The interaction was not statistically significant ($P > .05$). There were statistically significant effects associated with litters and colours at the 1% leval. Sex effects were negligible and not significant.

Table 26

Analysis of Variance of yellow band length for Matings Mi.1-5

Source	d.f.	ss	MS	F	P
Total	45	46529.85			
Mean	1	45839.67			
Bet. subclasses	35	46477.43			
Error	10	52.42	5.24		
R (μ, l,s,c)	14	46365.28			
Error + Interaction	31	164.57	5.31		
Interaction	21	112.15	5.34	1.02	NS
R (μ,l,s)	13	46166.14			
Colour	1	199.14	199.14	37.50	**
R (μ,l,c)	13	46364.26			
Sex	1	1.02	1.02	.19	NS
R (μ,s,c)	3	46042.30			
Litters	11	322.98	29.36	5.53	**

Table 26 indicated that there were highly significant differences in yellow band length between the means of the dark and light mice groups. The least squares estimates of effects are shown in Table 27.

Table 27

Estimates of effects for litters, sex and colours on yellow band length for Matings Mi.1-5.

Item	Effect
l_1	- 6.25
l_2	2.24
l_3	- .50
l_4	- .45
l_5	- .49
l_6	3.03
l_7	- .14
l_8	4.64
l_9	- .40
l_{10}	- 1.85
l_{11}	- .68
l_{12}	.46
s_1	- .17
s_2	.17
c_1	- 2.25
c_2	2.25
μ	31.44

Table 27 shows that the effect of the dark genotype (d/+,A/a) on the yellow band was a reduction in length of 4.50 cms. as measured at a magnification of X500. However, this does not preclude the possibility that the dark gene may have darkening effects on the mouse coat other than by a reduction of the yellow band lengths. Again, these

results refer only to zigzag hairs, from the particular sampling position.

In an attempt to see if yellow band length could be used to classify mice as d/+,A/a, or +/+,A/a genotypes, the yellow band length measurements of all these 45 mice were adjusted for their respective litter effects. The overall mean of the yellow band lengths of all these mice with litter effects removed was 31.71. This value was taken as the mid-point between the means of the dark and light groups with litter effects removed and the number of dark mice above this value and the number of light mice below it were recorded. On the basis of yellow band length adjusted for litter effects, and taking the overall mean of these values as the mid-point between groups, 5 mice were classified differently from the visual classification, giving approximately 11% disagreement between the two methods.

The ratio of dark:light on the basis of the adjusted yellow band lengths was 21:24, which does not differ significantly from 1:1.

Yellow band lengths were corrected for litter, sex and colour effects, and the resulting estimates were used to obtain means for mice of the A^+/a, A^w/a, A^+/a^+, and A^w/a^t genotypes. These means are shown in Table 28.

Table 28

Means of yellow band lengths corrected for litter, sex and colour effects for the four Agouti genotypes from Matings Mi.1-5.

Genotype	A^+/a^t	A^+/a	A^w/a^t	A^w/a
Mean	31.44	31.75	30.47	31.50
S.E.	$\pm$ .54	$\pm$1.02	$\pm$ .68	$\pm$ .91
n	17	6	9	5

Table 28 was interpreted as indicating that the four agouti genotypes show no obvious differences in mean yellow band lengths, after correction for litter, sex and colour effects.

However, the $Mi^{wh}/+$ genotype was shown to have a marked effect on the yellow band length. In all $Mi^{wh}/+$ genotypes studied the yellow pigment began in the first medullary cell at the distal end of the medulla. That is, the sub-terminal band of black medullary pigment which is the first medullary pigment formed in the hair was absent. Consequently on an agouti background the $Mi^{wh}/+$ genotype causes a dilution of the coat colour by both reducing the size of the black medullary pigment clumps in the lower portion of the hair (Grobman and Charles 1947) and also removing the sub-terminal black pigment band so that the yellow band is the corresponding amount longer.

(iii) Discussion

The highly significant litter effects would suggest that classification of dark and light mice on the basis of yellow band length must be carried out on a within litter basis. Consequently, single values for dark and light mice cannot be used to classify other mice on the basis of yellow band length. No obvious explanation for the litter effects has been found. The litter estimates show no relationship with either mean litter body weight or litter size. As the 4 mating groups were combined in this analysis, some of the between litter variation may be due to the genetic backgrounds of the parents, or the maternal effects of the mothers. This point will be discussed when referring to the results of Matings MiT and U.1 which comprised one set of parents each.

That sex effects were negligible is in accordance with results reported by Dry (1928) who stated that "where the conformation of the

body is the same, no differences between the sexes has been detected in any hair character."

The value of 11% disagreement between objective and visual methods cannot be taken too seriously as the mean may not represent the mid-point between the two groups, the values obtained as the means of 8 hairs for each mouse will be subject to sampling variation, and the litter effects are probably not "fixed" in the statistical sense and hence are probably biased (Henderson 1948). The fact that some overlapping values occur with the use of yellow band length as a measure of phenotype, together with the absence of sufficiently large numbers of mice to test an expected bimodal distribution of yellow band lengths, indicates that the possibility of a continuous distribution of phenotype has not been disproved.

(iv) Conclusions

Dark mice of the genotype d/+, A/a, $Mi^{wh}/+$, show a reduction in length of the yellow agouti band (in the order of 4.50 cms when measured at a magnification of X500), as compared with mice of the genotype +/+, A/a, $Mi^{wh}/+$. This conclusion refers only to zigzag hairs from the sampling position.

Yellow band length used as the mean value of 8 hairs is still not a sufficiently exact measure of phenotype to prevent the occurrence of some overlapping phenotypic values. The ratio of 21:24 for dark:light mice on this criterion is in agreement with the expectation of 1:1.

The genotypes A^w/a, A^w/a^t, A+/a and A^+/a^t do not differ in mean band lengths. However, the $Mi^{wh}/+$ genotype causes the removal of the subterminal black band with a consequent increase in yellow band length.

Mean values of yellow lengths for the dark and light genotypes

cannot be used to classify other mice as light or dark, due to the presence of unexplained highly significant litter effects.

Although one effect of the dark gene has been found, the measure of this effect is not of great help in classification problems.

2. Mating MiT

(i) Methods of Analysis

Two analyses of variance models were used. They were:

(a) $y_{ijklm} = \mu + l_i + b_j + s_k + c_l + e_{ijklm}$

where $i = 1, 2.....5$

$j = 1,2$

$k = 1,2$

$l = 1,2$

$m = 0,1,2,3$

The l_i represented litter effects, the b_j the genetic background effects ($b_1 = Mi^{wh}/+$, $b_2 = +/+$) the s_k sex effects (s_1 = male, s_2 = female) and the c_l the colour classification effects (c_1 = very dark, c_2 = dark, c_3 = light). The y_{ijklm} were the lengths (as the mean of 8 zigzag hairs) of medulla from top of the medulla to the proximal end of the yellow band. Thus for $Mi^{wh}/+$ genotypes this was the length of the yellow band, while for +/+ genotypes this was the sum of the lengths of the subterminal black band length and the yellow band length.

(b) $y_{ijk} = \mu + l_i + c_j + e_{ijk}$

where $i = 1, 2 .... 5$

$j = 1, 2, 3$

$k = 0, 1 .....5$

The l_i represented litter effects and the c_j colour classification effects (c_1 = very dark, c_2 = dark, c_3 = light). The y_{ijk} were (i) Length of the subterminal black band (as the mean of 8 zigzag hairs) (ii) length of the yellow band (as the mean of 8 zigzag hairs). Both these analyses were carried out on the wild-type agouti offspring.

The least squares equations for model (a) are shown in Table 29. The least squares equations for model (b) are shown in Table 30.

Table 29

The least squares equations for the sum of yellow + black band lengths for offspring of Mating Mi.T.

$\mu+(l_1$	l_2	l_3	l_4	$l_5)$	b_1	b_2	s_1	s_2	c_1	c_2	c_3	Σy
$\hat{l}_1$ 13					6	7	7	6	0	9	4	364.60
l_2	11				7	4	5	6	0	9	2	275.18
l_3		10			7	3	5	5	2	6	2	252.30
l_4			7		4	3	4	3	0	6	1	216.00
l_5				7	2	5	5	2	5	1	1	172.43
b_1 6	7	7	4	2	26	0	14	12	2	20	4	682.35
b_2 7	4	3	3	5	0	22	12	10	5	11	6	598.16
s_1 7	5	5	4	5	14	12	26	0	5	17	4	691.84
c_1 0	0	2	0	5	2	5	5	2	7	0	0	150.12
c_2 9	9	6	6	1	20	11	17	14	0	31	0	805.90
c_3 4	2	2	1	1	4	6	4	6	0	0	10	324.49

Table 30

The least squares equations for black band length and yellow band length for the wild-type agouti mice of Mating Mi.T

$\mu +$	l_1	l_2	l_3	l_4	l_5	c_1	c_2	c_3	Black Band Length ξ_y	Yellow Band Length ξ_y
l_1	7					0	5	2	53.18	154.73
l_2		4				0	3	1	26.30	64.20
l_3			3			1	1	1	28.81	61.94
l_4				3		0	2	1	20.50	70.69
l_5					5	4	0	1	39.68	85.12
c_1	0	0	1	0	4	5	0	0	40.06	70.81
c_2	5	3	1	2	0	0	11	0	85.05	210.12
c_3	2	1	1	1	1	0	0	6	36.36	155.75

(ii) Results and Discussion

The analysis of variance of interactions and effects for the sum of black and yellow band lengths is shown in Table 31.

Table 31

Analysis of variance of interactions and effects for the sum of black and yellow band lengths of mice from Mating Mi.T

Source	d.f.	s.s.	M.s.	F	P
Total	48	35594.74			
Mean	1	34160.54			
Bet. subclasses	31	35255.52			
Error	17	339.22	19.95		
R (μ,l,b,s,c)	9	34891.51			
Error & Interactions	39	703.23	18.03		
Interactions	22	364.01	16.55	.83	N.S
R (μ,l,b,s)	7	34398.01			
Colours	2	493.50	246.75	13.69	* *
R (μ,l,b,c)	8	34884.42			
Sex	1	7.09	7.09	.39	N.S
R (μ,l,s,c)	8	34887.98			
Backgrounds	1	3.53	3.53	.20	N.S
R (μ,b,s,c)	5	34723.66			
Litters	4	167.85	41.96	2.33	.05-.10

The interactions were not significant. Colour effects were significant at the 1% level, while effects due to sex and background were negligible, and not significant. The litter effects showed a probability of about 7% of having arisen by chance, and consequently were not significant at 5% level.

The analysis of variance of interactions and effects for black band lengths and yellow band lengths are shown in Tables 32 and 33 respectively, which are based on the results from wild-type agouti mice only.

Table 32

Analyses of variance of interactions and effects for black band lengths from wild-type agouti mice of Mating Mi.T.

Source	d.f.	s.s.	M.S.	F.	P.
Total	22	1224.09			
Mean	1	1185.11			
Bet. subclasses	11	1207.49			
Error	11	16.60	1.51		
R (μ,l,c)	7	1204.02			
Error & Interactions	15	20.07	1.34		
Interactions	4	3.47	.87	.58	N.S.
R (μ,l)	5	1190.47			
Colours	2	13.55	6.77	5.05	*
R (μ,c)	3	1198.91			
Litters	4	5.11	1.28	.96	N.S

Interactions were non-significant. The colour effects were significant at the 5% level. Litter effects were non-significant.

Table 33

Analyses of variance of interactions and effects for yellow band lengths of the wild-type agouti mice from Mating Mi.T

Source	d.f.	s.s.	M.S.	F.	P.
Total	22	9458.14			
Mean	1	8667.70			
Bet. subclasses	11	9249.42			
Error	11	208.72	18.98		
R (μ,l,c)	7	9184.62			
Error & Interactions	15	273.52	18.23		
Interactions	4	64.80	16.20	.85	N.S.
R (μ,l)	5	8844.19			
Colours	2	340.43	170.21	9.34	*
R (μ, c)	3	9059.48			
Litters	4	125.14	31.28	1.72	N.S.

The interactions were not significant. Colour effects were significant at the 5% level. Litter effects were not significant.

The results of the Mi.1-5 matings showed that one of the effects of the $Mi^{wh}/+$ genotype was to remove the subterminal black band and consequently make the yellow band this much longer at the distal end. Hence it became of interest to see, apart from this effect, whether the $Mi^{wh}/+$ genotype had any other effect on yellow band length. The analysis of variance shown in Table 31 indicates that the difference between the $Mi^{wh}/+$ and $+/+$ backgrounds was non-significant. That is, yellow band lengths of $Mi^{wh}/+$ mice do not differ significantly from the sums of sub-terminal black band length and yellow band length for $+/+$ mice. More

concisely, the distance from the tip of the medulla to the proximal end of the yellow band is the same in both $Mi^{wh}/+$ and +/+ genotypes, the only difference being that in the latter genotype, the distal region of this segment is black, while in the former the whole segment is yellow.

Table 31 also indicates that significant differences existed between the three dark phenotypes for the length of this segment from the tip of the medulla to the proximal end of the yellow band. Consequently the dark genotypes had significant effects on the length of this segment, which were the same in both $Mi^{wh}/+$ and +/+ genotypes.

However, the actual length of yellow pigment in this segment for wild-type agouti mice depends on the length of the sub-terminal black band at the distal end of the medulla. Table 32 indicates that significant differences existed between the three dark phenotypes for the length of this black band.

In view of the possibility that the differences between dark phenotypes in black band lengths and the sums of black band length and yellow band length of wild-type agouti mice might be compensating, the analysis of variance of yellow band lengths only, as shown in Table 33, was carried out. Differences in yellow band lengths were significant at the 5% level between the three dark phenotype groups.

A general picture of the relationships between black length, yellow length and the sum of these, as far as the dark phenotypes are concerned, on both $Mi^{wh}/+$ and +/+ backgrounds, is shown in Table 34. This Table shows the estimates of $\mu + c_i$ as derived from the analyses of variance. The exact figures should not be taken too seriously as the estimates were obtained with different statistical models and two different populations of mice. This is shown by the fact that the sum of the effects for black lengths and yellow lengths do not add up exactly to the estimate of the effect for the sum of black and yellow lengths.

Table 34

Estimates of $\mu + c_i$ for black band lengths, yellow band lengths, and the sum of these, for all mice from Mating Mi.T

	Black length		Yellow length		Black + Yellow length	
Phenotype ↓	$Mi^{wh}/+$	+/+	$Mi^{wh}/+$	+/+	$Mi^{wh}/+$	+/+
Very dark	0	7.2	20.8	12.3	20.8	20.8
Dark	0	7.9	26.5	19.9	26.5	26.5
Light	0	6.0	33.0	25.9	33.0	33.0

From the estimates in Table 34 and the analyses of variance the following conclusions may be formed.

(i) In wild-type agouti mice, the dark and very dark groups showed significant reductions in the length of the yellow band as compared with the light group, with the very dark group showing the greatest reduction. There were significant differences between the three groups in the black band length, although the estimates of effects were not related to the order light → dark → very dark. However, both the dark and very dark groups showed more black pigment than the light group.

(ii) In $Mi^{wh}/+$ mice the black band is replaced by yellow pigment. Apart from this, the estimates of yellow length for each of the three dark groups are the same as those for yellow + black lengths of wild-type agouti mice from the same groups.

(iii) The yellow band lengths of $Mi^{wh}/+$ mice (or black + yellow lengths of +/+ mice) were significantly reduced in the dark and very dark groups as compared with the light group with very dark mice showing the greatest reduction.

(iv) Consequently the dark genotypes have effects on the yellow band lengths of $Mi^{wh}/+$ mice, and on the yellow and black pigment bands of +/+ mice.

Tables 31, 32 and 33 indicate that the litter effects on yellow band lengths and black band lengths of wild-type agouti mice, and on the sum of these for both $Mi^{wh}/+$ and +/+ mice, were not statistically significant at the 5% level. As these litters came from the one parent pair, a large portion of the highly significant litter effects obtained from Matings Mi.1 - 5 may have been due to differences between parent pairs (genetic effects) or differences between mothers (permanent maternal effects).

(III) Conclusions

In wild-type agouti mice, the dark genotypes d/+, A^w/a^t and d/d A^wA^w were shown to cause a significant reduction in length of the yellow agouti band as compared with "normal" or light mice with the genotype d/+ A^wA^w, while the very dark genotype d/d,A^wa^t caused an even greater reduction. Both very dark and dark mice also showed a significant increase in length of the subterminal black band as compared with light mice. The increase in black length and the decrease in yellow length were not compensating and so the sum of these two lengths was reduced in dark mice and still further reduced in very dark mice. Consequently the dark genotypes have a dual effect on the length of yellow pigment in the agouti hair, both decreasing the length of yellow pigment and increasing the length of black pigment.

The dark genotypes have the same effects in Dominant White ($Mi^{wh}/+$) agoutis as in wild-type agoutis, except that in the $Mi^{wh}/+$ mice the black pigment band has been replaced by yellow pigment.

It was concluded that real differences existed in the lengths of yellow pigment between the three visual phenotypic groups.

As litter effects were not significant in this study, it is probable that much of the variation between litters in Matings Mi.1-5 was due to differences between parent pairs or mothers. Consequently between litter comparisons of dark phenotypes (visual or yellow band lengths) may be possible for full-sibs.

3. MATING U.1

(i) Methods of analysis

The data were analysed by use of the analysis of variance model

$$y_{ijkl} = \mu + l_i + s_j + c_k + \varepsilon_{ijkl}$$

where $i = 1,2,3$

$j = 1,2$

$k = 1,2$

$l = 0,1,2$

The l_i are litter effects, the s_j sex effects (s_1 = male and s_2 = female) and the c_k colour classification effects (c_1 = very dark, c_2 = dark.). The y_{ijkl} were:

(a) Yellow band length (mean value of eight hairs)

(b) Black band length (mean value of eight hairs)

(ii) Results and Discussion

The least squares equations are shown in Table 35. The analyses of variance of interactions and effects are shown in Tables 36 (yellow band length) and 37 (black band length).

Table 35

The least squares equations for yellow band lengths and black band lengths of the first 14 mice born to Mating U.1

$\mu +$	$(l_1$	l_2	$l_3)$	s_1	s_2	c_1	c_2	Yellow Band length Σy	Black Band length Σy
l_1	5			2	3	2	3	55.93	43.80
l_2		4		3	1	2	2	72.90	28.63
l_3			5	1	4	2	3	81.75	41.00
s_1	2	3	1	6	0	2	4	94.78	42.82
s_2	3	1	4	0	8	4	4	115.80	70.61
c_1	2	2	2	2	4	6	0	77.05	53.57
c_2	3	2	3	4	4	0	8	133.53	60.06

Table 36

Analyses of variance of interactions and effects for yellow band lengths of mice from Mating U.1

Source	d.f.	s.s.	M.S.	F.	P.
Total	14	3383.32			
Mean	1	3167.42			
Bet. subclasses	10	3367.10			
Error	4	16.22	4.05		
R (μ,l,s,c)	5	3353.35			
Error & Interactions	9	29.97	3.33		
Interactions	5	13.75	2.75	.68	N.S.
R (μ,l,s)	4	3291.53			
Colours	1	61.82	61.82	18.56	**
R (μ,l,c)	4	3352.39			
Sex	1	.96	.96	.29	N.S
R (μ,s,c)	3	3219.88			
Litters	2	133.47	66.73	20.04	**

Table 37

Analyses of variance of interactions and effects for black band lengths of mice from Mating U.1

Source	d.f.	s.s.	M.S.	F.	P.
Total	14	951.56			
Mean	1	919.03			
Bet. subclasses	10	946.90			
Error	4	4.66	1.16		
R (μ,l,s,c)	5	936.80			
Error and Interactions	9	14.76	1.64		
Interactions	5	10.10	2.02	1.74	N.S.
R (μ,l,s)	4	931.76			
Colours	1	5.04	5.04	3.07	N.S.
R (μ,l,c)	4	932.54			
Sex	1	4.26	4.26	2.60	N.S.
R (μ,s,c)	3	933.10			
Litters	2	3.70	3.70	1.65	N.S.

Data were collected only on mice from the first three litters of Mating U.1, that was, the first 14 mice born.

The analysis of variance of yellow band lengths shown in Table 36 shows that the visual segregation of very dark wild-type agouti ($d/d,A^w/a^t$) and dark wild-type agouti ($d/d,A^w/A^w$) mice in Mating U.1 was also accompanied by statistically significant differences in yellow band lengths between the two genotypes. The estimates of colour effects show that the yellow band lengths of $d/d,A^w/a^t$ mice were reduced by 4.4 cms (at X500) as compared with the yellow band lengths of $d/d,A^w/A^w$ mice.

Differences between litter means were statistically significant. As all the 14 mice were born to the same parent pair, these litter effects remain unexplained.

Table 37 indicates that no statistically significant differences between colours, litters and sexes were observed for black band lengths. As differences between very dark and dark phenotypes were tested here, compared with very dark, dark and light phenotypes in Mating Mi.T, the absence of significant differences in black band lengths between colours cannot be considered a contradiction to the Mi.T results which did show significant differences.

4. EFFECTS OF THE DARK GENOTYPES ON THE LENGTH OF YELLOW PIGMENT.

(i) Introduction

In order to see if the results of the objective studies supported the conclusions obtained from the visual studies in regard to the hypothesized genotypes of the very dark, dark and light phenotypes from Matings Mi.1-5, Mi.T and U.1, the estimates of $\mu + c_i$ for (black ~~and~~ yellow) band lengths were compared for all matings and all phenotypes. The use of the $\mu + c_i$ for the comparisons gave adjustment for all other effects including the random error term. Using the estimates of (black ~~and~~ yellow) band lengths allowed $Mi^{wh}/+$ and $+/+$ genotypes to be considered together.

On the basis of these comparisons it was also hoped to obtain some method of separating the two dark genotypes ($d/+$, A^w/a^t and $d/d, A^w/A^w$) which were classified together in Mating Mi.T.

(ii) Results and Discussion

The estimates of $\mu + c_i$ are shown in Table 38

Table 38

Estimates of $\mu + c_i$ for (yellow + black) band lengths, for the visual phenotypic groups of Matings Mi.1-5, Mi.T and U.1

Mating		Very Dark	Dark	Light
Mi.1-5	Genotype	-	$d/+, A/a$	$+/+, A/a$
	$\mu + c_i$	-	29.2	33.7
Mi.T	Genotype	$d/d, A^w/a^t$	$d/d, A^w/A^w$ $d/+, A^w/a^t$	$d/+, A^w/A^w$
	$\mu + c_i$	20.8	26.5	33.0
U.1	Genotype	$d/d, A^w/a^t$	$d/d, A^w/A^w$	-
	$\mu + c_i$	20.2	25.8	-

The actual figures shown in Table 38 should not be considered too seriously. These estimates were obtained from different groups of mice, with different numbers in each group, and by the use of different analysis of variance models. However, the estimates show extremely good agreement with, and support for, the conclusions formed from the visual studies. In spite of a few mis-classifications, the estimates for the light mice ($d/+,A^w/A^w$ and $+/+,A/a$) from Matings Mi.1-5 and Mating Mi.T show excellent agreement; as do the estimates for the very dark mice ($d/d,A^w/a^t$) from Matings Mi.T and U.1. The estimates for dark mice ($d/d,A^w/A^w$ and $d/+,A^w/a^t$) provide evidence in support of the hypothesis that the dark mice from Mating Mi.T are comprised of two genotypic groups; the value of 26.5 for approximately equal numbers of $d/d,A^w/A^w$ and $d/+,A^w/a^t$ mice from Mating Mi.T lying between that of 29.2 for $d/+,A/a$ mice from Matings Mi.1-5, and 25.8 for $d/d,A^w/A^w$ mice from Mating U.1.

Although the estimates in Table 38 may not be truly accurate or comparable, it was decided to accept them as being true estimates for the respective genotypes, in order to obtain some information on the full 1:1:1:1 segregation in Mating Mi.T. The estimates accepted were those from the U.1 mating and Matings Mi.1-5 which were obtained independently of Mating Mi.T. The mid-points between estimates were chosen as representing the mid-points between genotypes, as follows:

Genotype	$\mu + c_i$	Mid-Value
$d/d,A^W/a^t$	20.2)	23.0
$d/d,A^W/A^W$	25.8)	
$d/+,A^W/a^t$	29.2)	31.4
$d/+,A^W/A^W$	33.7)	

The values of (yellow + black) band lengths of all mice in Mating Mi.T were then adjusted for litter, sex and background effects as estimated by the model used in that Mating. All mice of the A^W/a^t genotype with adjusted values falling below 23.0 were classified as being of the $d/d,A^W/a^t$ genotype. Mice of the A^W/A^W genotype with adjusted values above 31.4 were classified as the $d/+,A^W/A^W$ genotype. All other mice were classified into two groups according to whether they were of the A^W/A^W or A^W/a^t genotypes, and the mean of the adjusted values calculated for both groups. The mean values were:

$$A^W/A^W = 26.2$$

$$A^W/a^t = 27.6$$

which are in as reasonable agreement with the values of 25.8 and 29.2 as might be expected, on the basis of such an approximate procedure. The numbers of mice in the four genotypic groups, on the basis of this classification, were:

$d/d,A^W/a^t$	- 9
$d/d,A^W/A^W$	- 11
$d/+,A^W/a^t$	- 15
$d/+,A^W/A^W$	- 13
Total	48

The ratio of 9:11:15:13 is in good agreement with a 1:1:1:1 ratio although the figures were not considered to be sufficiently dependable

to warrant a Chi-square analysis.

(iii) Conclusions

By making some rather vast assumptions, evidence has been provided from the objective studies to suggest that dark genotypes expected on the basis of the visual results to be the same in different matings, are in fact the same. The yellow band length studies also provide evidence in support of the hypothesis that the dark mice of the Mi.T mating consist of approximately equal numbers of two dark genotypes. On the basis (black + yellow) band lengths the order of genotype-phenotype relations are:

$d/+\ A^w/A^w$ and $+/+\ A^w/+ \rightarrow d/+, A^w/a^t \rightarrow d/d, A^w/A^w \rightarrow d/d, A^w/a^t$

in order of decreasing band lengths. These results are in agreement with results of the visual studies.

It is also likely that the segregation of all four dark genotypes in Mating Mi.T was in agreement with the expectation of 1:1:1:1.

5. PLEIOTROPIC STUDIES OF THE DARK GENE

(i) Introduction

The following sections describe methods used and the results obtained, in an attempt to obtain some knowledge of the physiological relationships between causal factors underlying the reduction in yellow band lengths of dark mice.

The mice used in this study were the Dominant White agoutis ($Mi^{wh}/+,A/a$) from Matings Mi.1-5, as these represented a total of 45 mice among which the dark:light segregation was fairly easily observed. Time did not permit full analysis of all data from the other matings and consequently the results obtained refer only to the dark genotype $d/+$, A/a, $Mi^{wh}/+$, and the light genotype $+/+,A/a.Mi^{wh}/+$.

(ii) Methods of analysis

The techniques of the analyses of variance and covariance were used in this study. The analysis of variance model was

$$y_{ijkl} = \mu + l_i + s_j + c_k + e_{ijkl}$$

where (a) $i = 1,2 \ldots\ldots 12$

$j = 1,2$

$k = 1,2$

$l = 0,1,2$

(b) $i = 1,2\ldots 7$

$j = 1,2$

$k = 1,2$

$l = 0,1,2$

The l_i and litter effects, the s_j sex effects (s_1 = male, s_2 = female), and the c_k colour classification effects (c_1 = dark, c_2 = light).

The model was analysed by the method previously described. The analyses of covariance were based on the analyses of variance as previously described.

The y_{ijkl} for (a) were:

(i) Yellow band length (designated Y)

(ii) The length from the tip of the hair to the distal constriction (O-1)

(iii) The length from the tip of the hair to the distal end of the yellow band, i.e. the length of solid cortex (G)

(iv) The total length of black medullary pigment in a 5 cm. strip of hair taken mid-way between the first and second constrictions from the hair tip (B)

(v) The number of yellow cells in the yellow band (Y_c)

(vi) The number of medullary cells in the 5 cm. hair strip described above (L)

(vii) Body weight at 21 days of age (W)

The y_{ijkl} for (b) were:

(i) Yellow band length (Y)

(ii) Total hair length (H)

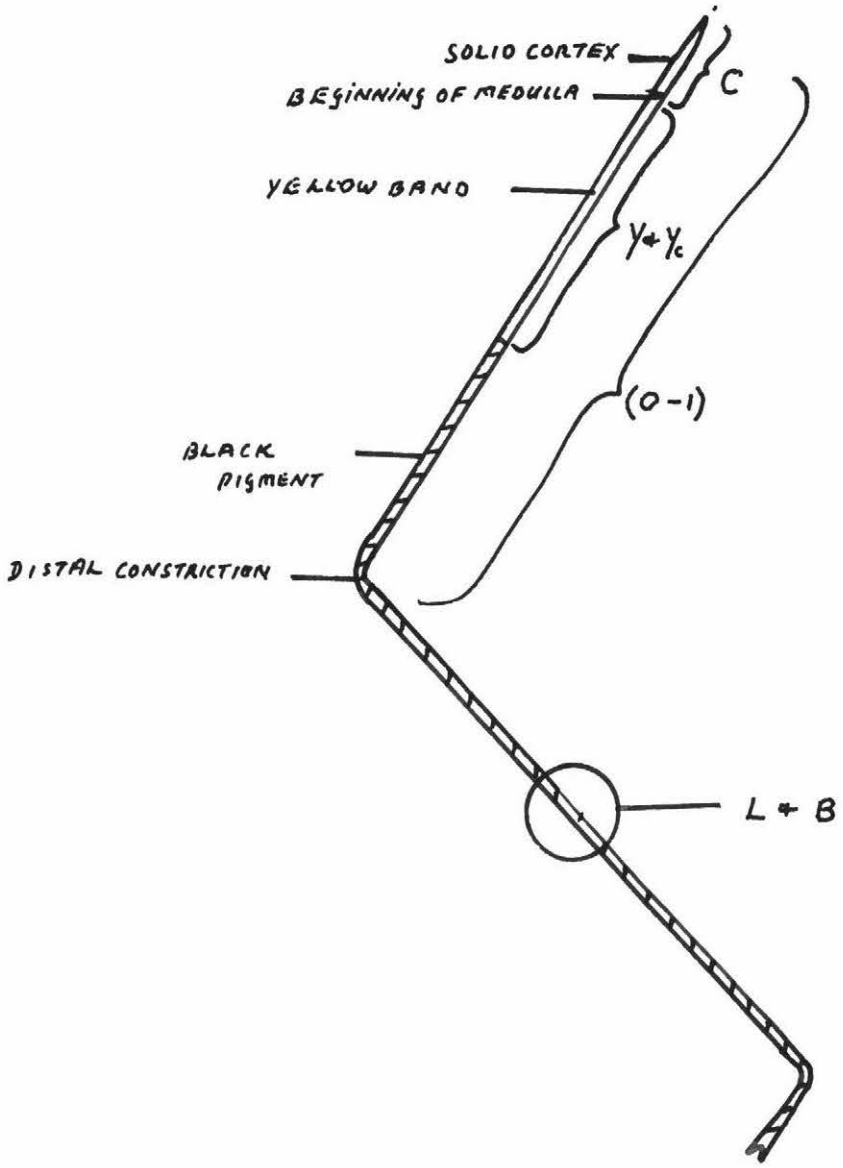


FIG. 8.

DISTAL REGION OF A $Mi^{wl}/+$ ZIGZAG HAIR SHOWING THE POSITIONS OF Y, Y_c, $(O-1)$, L, + B.

The need for model (b) resulted from hair lengths of mice from litters 1-5 being estimated in terms of projection microscope field diameters. In preference to attempting conversion of these values to terms of "cms at X86" which might have introduced large errors, the data on total hair length for these mice were discarded. Consequently the mice used with model (b) were those from litters 6-12. The data on Y for these mice were re-analysed by model (b) to allow the covariance analysis for Y and H to be carried out.

The relations between Y, N, C, (O-1), B and L can be seen in Figure 8.

(iii) Results

The least squares equations for models (a) and (b) are shown in Table 25. The $\underset{\sim}{\varepsilon}_y$ vectors for each variable for these equations are shown in Table 39.

Table 39

The R.H.S. of the least squares equations shown in Table 25

Equation	Model (a)						Model (b)
μ	(0-1)	C	B	Y_c	L	W	H
l_1	286.87	62.81	91.31	216.86	44.25	39.8	-
l_2	147.13	30.19	39.88	151.74	22.37	24.0	-
l_3	304.43	60.67	80.12	256.12	41.88	40.8	-
l_4	214.12	47.19	61.25	198.50	30.50	29.6	-
l_5	148.49	31.56	41.19	137.87	22.25	16.8	-
l_6	525.92	108.06	154.74	534.24	85.88	53.8	362.72
l_7	462.74	94.55	123.41	409.86	71.30	51.4	322.00
l_8	307.25	62.43	76.00	323.36	48.00	34.6	225.57
l_9	195.81	41.80	53.69	217.87	32.19	24.8	141.87
l_{10}	224.87	44.75	64.50	211.62	36.18	31.6	163.06
l_{11}	146.99	31.50	34.12	142.25	22.56	21.0	109.37
l_{12}	365.72	76.93	92.49	366.36	57.50	47.8	267.36
s_1	1399.14	290.91	392.67	1341.21	217.55	185.2	693.23
s_2	1931.20	401.53	520.03	1825.44	297.31	230.8	898.72
c_1	1472.09	303.35	429.04	1310.59	231.25	183.6	677.05
c_2	1858.25	388.09	483.66	1856.06	283.61	232.4	914.90

The ξ_j for Y is shown in Table 25 for both models (a) and (b). The analysis of variance for Y using model (a) is shown in Table 26, and has been discussed in that section.

In order to see whether the reduction of yellow length in dark mice took place at the distal or proximal end of the band relative to the tip of the hair, the analysis of variance on the solid cortex lengths was carried out as shown in Table 40.

Table 40

Analyses of variance of interactions and effects on the length of solid cortex for all $Mi^{wh}/+$ mice of Matings Mi.1-5.

Source	d.f.	s.s.	M.s.	F.	P.
Total	45	10687.88			
Mean	1	10654.96			
Bet. subclasses	35	10679.66			
Error	10	8.22	.82		
R (μ,l,s,c)	14	10667.43			
Error & Interactions	31	20.45	.66		
Interactions	21	12.23	.58	.71	N.S.
R (μ,l,s)	13	10664.62			
Colours	1	2.81	2.81	4.26	*
R (μ,l,c)	13	10667.06			
Sex	1	.37	.37	.56	N.S.
R (μ,s,c)	2	10679.66			
Litters	12	13.05	1.19	1.80	N.S.

Table 40 indicates that only colour effects were statistically

significant at the 5% level. The estimate of the colour difference was .52 cms, the dark genotype being the shorter.

An analysis of covariance for colour effects was then carried out with yellow length as the dependant variate and solid cortex length as the independant variate. This analysis is shown in Table 41.

Table 41

Analysis of covariance with yellow length as the dependant variate (y) and solid cortex length as the independant variate (x) for all $Mi^{wh}/+$ mice from Matings Mi.1-5.

Source	c.ss.y	c.ss.xy	c.ss.x	b	b.ss	Dev.ss	d.f	M.S.	F.	P.
Total	690.18	41.92	32.92							
R(l,s,c)	525.61	20.24	12.47							
Residual	164.57	21.68	20.45	1.06	22.98	141.59	30	4.72	4.72	*
Colours	199.14	23.39	2.81							
Colours + Residual	363.71	45.07	23.26	1.94	87.44	276.27				
Colours (adj.)						134.68	1	134.68	28.53	**

The analysis of covariance shown in Table 41 indicates that the regression coefficient (b) in the residual line was significant at the 5% level, and consequently adjustments of yellow lengths for the linear within sex within litter within colour regression on solid cortex length were worth carrying out. The adjusted sums of squares for colours was still significant at the 1% level. It was concluded that the dark geno-

type reduced yellow band length by causing a small but significant reduction in cortex length which shows a significant positive within subclass linear regression with yellow band length, and also by causing a direct reduction on yellow band length. Relative to the tip of the hair, the estimates of the dark effects for C and Y (i.e. -.52 and -4.50 cms. respectively below the values for the light genotype) show that the dark genotype reduces yellow band length by about 5.02 cms. at the proximal end of the band.

As the dark genotype caused significant reductions in the length of the solid cortex segment and yellow band length, it appeared as if the dark genotype might reduce hair length generally. Analyses of variance were thus carried out on both the length of the distal hair segment (0-1) and the total hair length from the tip of the hair to the base of the "club" bulb. These analyses are shown in Tables 42 and 43.

Table 42

Analyses of variance of interactions and effects for the variable (0-1) for all $Mi^{wh}/+$ mice of Matings Mi.1-5.

Source	d.f.	s.s.	M.S.	F.	P.
Total	45	247338.63			
Mean	1	246470.32			
Bet. subclasses	35	247191.32			
Error	10	147.31	14.73		
R (μ,l,s,c)	14	246879.72			
Error & Interactions	31	458.91	14.80		
Interactions	21	311.60	14.34	1.01	N.S.
R (μ,l,s)	13	246863.59			
Colours	1	16.13	16.13	1.09	N.S.
R (μ,l,c)	13	246874.50			
Sex	1	5.22	5.22	.35	N.S.
R (μ,s,c)	3	246492.72			
Litters	11	387.00	35.18	2.38	*

Table 43

Analyses of variance of interactions and effects for total hair length of $Mi^{wh}/+$ mice from litters 6 to 12 of Matings Mi.1-5

Source	d.f.	s.s.	M.s	F.	P
Total	30	84748.83			
Mean	1	84476.82			
Bet. subclasses	22	84728.31			
Error	8	20.52	2.56		
R (μ,l,s,c)	9	84685.61			
Error & Interactions	21	63.22	3.01		
Interactions	13	42.70	3.28	1.28	N.S.
R (μ,l,s)	8	84655.79			
Colours	1	29.82	29.82	9.91	**
R(μ,l,c)	8	84668.79			
Sexes	1	16.82	16.82	5.59	*
R (μ, s,c)	3	84505.75			
Litters	6	179.86	29.98	9.96	**

c_1 effect = -1.06 cms. (X86 mag.)

No significant differences were observed between dark and light mice or between sexes for the variable (O-1). Litter effects were just significant at the 5% level. Total hair length (H) showed significant differences between colours and litters at the 1% level and between sexes at the 5% level, both the dark genotype and females showing reductions.

In the absence of any data on length-time relations between mice during the growing period, it cannot be established whether the reduction in hair length of the dark genotype is a consequence of a reduced rate of hair growth, or a reduced time period of hair growth in these mice. Although the dark genotype reduced total hair length, the length of the distal hair segment (O-1) was not reduced. This suggests that, on a between colours basis, the distal constriction occurs after a fixed quantity of hair has grown, or a fixed time period of growth has occurred.

In an attempt to see whether the shorter hairs of the dark genotype might be a result of shorter medullary cells, an analysis of variance was carried out on the number of cells in the 5 cm. (X500 mag.) segment midway between the first and second distal constrictions (L). Thus L was used as an estimate of medullary cell length in this region of the hair. The analysis is shown in Table 44.

Table 44

Analysis of variance of interactions and effects for the variable L for all $Mi^{wh}/+$ mice from Matings Mi.1-5

Source	d.f.	s.s.	M.s.	F.	P.
Total	45	5917.09			
Mean	1	5890.68			
Bet. subclasses	35	5916.10			
Error	10	.99	.10		
R (μ,l,s,c)	14	5910.33			
Error & Interactions	31	6.76	.22		
Interactions	21	5.77	.27	2.70	N.S.
R (μ,l,s)	13	5909.88			
Colours	1	.45	.45	2.04	N.S.
R (μ,l,c)	13	5910.19			
Sex	1	.14	.14	.62	N.S.
R (μ,s,c)	3	5893.01			
Litters	11	17.32	1.57	7.14	**

Table 44 indicates that there were no statistically significant differences between dark and light genotypes for L. It was concluded that the shorter hair lengths of dark mice were not due to these mice having reduced medullary cell lengths as measured by L. Litter effects

were significant at the 1% level.

As the reduction in yellow band lengths of dark mice might have been a consequence of the reduction in hair length of these mice, an analysis of covariance was carried out for colour effects with yellow length as the dependant variate (y) and the total hair length as the independant variate (x). As the analysis of variance of hair lengths was carried out on mice from litters 6 to 12, an analysis of yellow lengths was carried out for these same mice to enable the covariance analysis to be performed. The results of the analysis on yellow length are shown in Table 45, and are in good agreement with those obtained from the analysis using all mice shown in Table 26. The analysis of covariance is shown in Table 46.

Table 45

Analysis of variance of interactions and effects for yellow band lengths of $Mi^{wh}/+$ mice from litters 6 - 12 of Matings Mi.1-5.

Source	d.f.	s.s.	M.S.	F.	P.
Total	30	32674.41			
Mean	1	32401.93			
Bet. subclasses	22	32631.14			
Error	8	43.27			
R (μ,l,s,c,)	9	32578.12			
Error & Interactions	21	96.29	4.59		
Interactions	13	53.02	4.08		
R (μ,l,s)	8	32499.43			
Colours	1	78.69	78.69	17.14	**
R (μ,l,c)	8	32578.78			
Sexes	1	0	0	0	N.S.
R (μ,s,c)	3	32464.17			
Litters	6	113.95	18.99	4.14	**

c_1 effect = - 1.74 cms (X500 mag.)

Table 46

Analysis of covariance on colour effects with yellow band length as the dependant variate (y) and total hair length as the independant variate (x) for $Mi^{wh}/+$ mice from litters 6 to 12 of Matings Mi.1-5

Source	c ss y	cssxy	cssx	b	bss	Dev.ss	d.f.	MS	F	P
Total	272.48	109.97	272.01							
R (1,s,c)	176.19	78.31	208.59							
Residual	96.29	33.24	63.42	.524	17.42	78.87	20	3.94	4.42	*
Colours	78.69	47.97	29.82							
Colours + Residual	174.98	81.21	93.04	.873	70.88	104.10				
Colours (adj)						25.23	1	15.23	6.40	*

The regression coefficient in the residual line of Table 45 was significant at the 5% level, and consequently adjustment of yellow band length for the linear within subclasses regression on total hair length was proceeded with. The results show that after allowing for this relationship, differences between colours were still significant at the 5% level. It was concluded that the dark genotype reduces yellow length as a consequence of reducing hair length, and also has a direct effect on the reduction of yellow length.

An analysis of covariance for colour effects was carried out with yellow band length as the dependant variate (y) and the number of yellow medullary cells as the independant variate (x). The analysis of

variance on the number of yellow cells is shown in Table 47(a) and the analysis of covariance in Table 48(a).

Table 47(a)

Analyses of variance of interactions and effects for the numbers of yellow medullary cells of all $Mi^{wh}/+$ mice from Matings M.1-5

Source	d.f.	s.s.	M.s.	F.	P
Total	45	226600.24			
Mean	1	222837.16			
Bet. subclasses	35	226455.75			
Error	10	144.49	14.45		
R (μ, l, s, c)	14	225857.87			
Error & Interactions	31	742.37			
Interactions	21	597.88	28.47	1.97	N.S.
R (μ, l, s)	13	224983.47			
Colours	1	874.40	874.40	30.71	**
R (μ, l, c)	13	225837.06			
Sexes	1	20.81	20.81	1.44	N.S.
R (μ, s, c)	3	224223.43			
Litters	11	1634.44	148.58	10.28	**

Table 48(a)

Analysis of covariance on colour effects with yellow length as the dependant variate (y) and number of yellow cells as the independant variate (x) for all $Mi^{wh}/+$ mice from Matings Mi.1-5.

Source	cssy	cssxy	cssx	b	bss	Dev.ss	d.f.	M.S.	F.	P
Total	690.18	1454.31	3763.08							
R ∈ (l,s,c)	525.61	1208.94	3020.71							
Residual	164.57	245.37	742.37	.331	81.22	83.35	30	2.78	29.22	**
Colours	199.14	416.51	874.40							
Colours + Residual	363.71	661.88	1616.77	.409	270.71	93.00				
Colours (adj.)						9.65	1	9.65	3.47	N.S.

As the mean squares for interactions and error in the analysis of variance of numbers of yellow medullary cells differed by approximately twice the error mean square, the mean square for error was used as a basis for testing the significance of effects. Both colour and litter effects were statistically significant at the 1% level. Sex effects were non-significant. These results are in agreement with those obtained from the analysis of yellow band lengths. The within sub-classes regression in the residual line of the analysis of covariance shown in Table 48a was significant at the 1% level. Adjusting yellow band lengths for this regression removed the significant differences between colours for yellow band lengths. It was concluded that dark mice had shorter yellow bands as a consequence of having less yellow cells.

~~variance on the number of yellow cells is shown in Table 47, and the analysis of covariance in Table 48.~~

An analysis of variance was carried out on the estimates of black medullary pigment lengths from the 5 cm (X500 mag) drawings, previously described. The results of this analysis are shown in Table 47(b).

Table 47(b).

Analysis of variance of interactions and effects on the variable B for all $Mi^{wh}/+$ mice from Matings Mi.1-5.

Source	d.f.	s.s.	M.S.	F.	P
Total	45	18718.77			
Mean	1	18511.58			
Bet. subclasses	35	18700.18			
Error	10	18.59	1.86		
R (μ,l,s,c)	14	18662.99			
Error & Interactions	31	55.78	1.80		
Interactions	21	37.19	1.77	.95	N.S.
R (μ,l,s)	13	18639.11			
Colours	1	23.88	23.88	13.27	**
R (μ,l,c)	13	18657.41			
Sexes	1	5.58	5.58	3.10	N.S.
R (μ,s,c)	3	18584.19			
Litters	11	78.80	7.16	3.98	**

c_1 effect = +.76 mm (x500 mag)

Table 47 shows that significant differences existed between colours and litters at the 1% level. Sex effects were not significant. These results are in reasonable agreement with those obtained from Table 16. An analysis of covariance was carried out on colour effects with black medullary pigment length per 5 cm. section as the dependant variable and the number of medullary cells per 5 cm. section as the independant variable. This analysis is shown in Table 48(b).

Table 48(b).

Source	cssy	cssxy	cssx	b	bss	Dev.ss	d.f.	M.s.	F.	P
Total	207.19	22.87	26.41							
R / (l,s,c)	151.41	15.76	19.65							
Residual	55.78	7.61	6.76	1.126	8.57	47.21	30	1.57	5.46	*
Colours	23.88	2.55	.45							
Colours + Residual	79.66	10.16	7.21	1.409	14.31	65.35				
Colours (adj)						18.14	1	18.14	11.55	**

The regression coefficient in the error line was significant at the 5% level. After adjustment of black medullary pigment lengths per 5 cms. for the linear within subclasses regression on number of medullary cells per 5 cms. significant effects at the 1% level still existed for between colours. It was concluded that the dark genotypes show increased lengths of black pigment clumps in the region of the hair studied, and this increase was not brought about by increases in cell lengths of this region.

Consequently, it was concluded that the dark genotype showed a direct effect of increasing the amount or distribution of black pigment in medullary cells of the region studied.

The analysis of variance of weaning weights (in gms) is shown in Table 49.

Table 49

Analysis of variance of interactions and effects for weaning weights for all $Mi^{wh}/+$ mice from Matings Mi.1-5.

Source	d.f.	S.S.	M.S.	F.	P.
Total	45	3940.40			
Mean	1	3845.69			
Bet. subclasses	34	85.59			
Error	10	9.12	.91		
R (μ,l,s,c)	14	3908.48	4.83	4.69	*
Error & Interactions	31	31.92	1.03		
Interactions	21	22.8	1.09	1.20	N.S.
R (μ, l,s)	13	3908.45			
Colours	1	.03	.03	.03	N.S.
R (μ,l,c)	13	3902.29			
Sexes	1	6.19	6.19	6.01	*
R (μ,s,c)	7	3855.18			
Litters	11	53.30	4.85	4.71	**

s_1 effect = +.44 gms.

There were no statistically significant differences between the body weights of the dark and light mice groups. As might be expected, sex and litter effects WERE significantly different.

No significant relationships were evident between body weights and any hair or pigment character on a within litter, within sex, within colour basis.

(iv) Discussion and Conclusions

The results of the pleiotropic studies suggest that the dark mice differed from the "normal" or light mice by having shorter hairs and yellow band lengths, and longer black pigment clumps in the region studied. The reductions in yellow band lengths could not be explained by reductions in hair length, but were almost completely explained by the reductions in the number of yellow cells. The reduction in yellow band length occurred at the proximal end of the band, relative to the tip of the hair.

The reduction in hair lengths of dark mice was not accompanied by differences of mean cell size as measured mid-way between the two distal constrictions. Consequently the shorter hair length of dark mice was probably the result of these mice having less and not smaller medullary cells. That the yellow band length reductions in dark mice were rendered non-significant after adjusting for the number of cells in the band, supports this conclusion. Further, the estimates of the extent of reduction of solid cortex and yellow band length in dark mice did not appear large

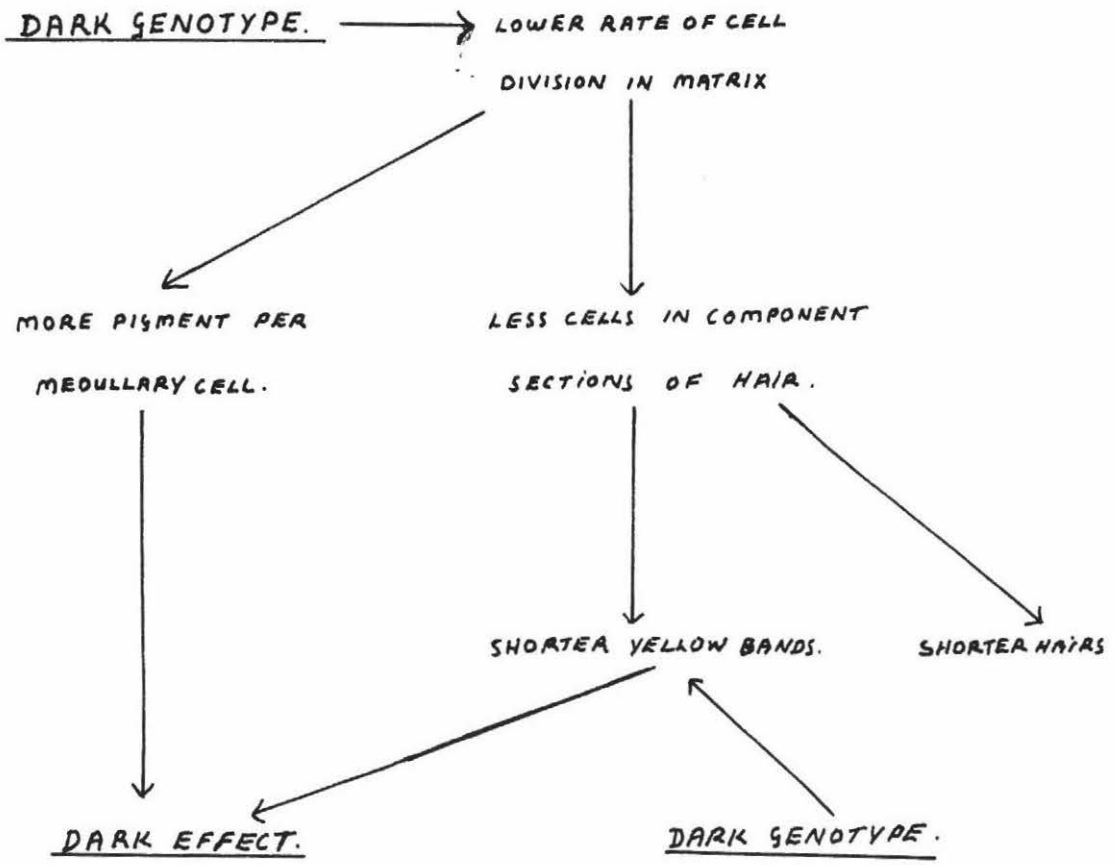


FIG. 9.

POSSIBLE PLEIOTROPY OF THE DARK GENOTYPE $Mi^{wh}/+$, $d/+$, A/a.

enough to account for the estimate of the reduction in hair length of these mice. Consequently the reduction in hair length was probably uniform throughout the hair. Such results could be explained by the hypothesis that the dark mice show reduced cell division rates in the matrix of the hair follicle. If the production of pigment granules by the melanocytes is not affected by the dark genotype, then a slower rate of cell division in the bulb might result in more granules entering each medullary cell. The results of the analyses of mean length of black pigment per cell in the region studied (as estimated by total length of black pigment in a 5 c.m. (X500 mag.) strip), provide evidence that this might be the case.

Such a scheme is shown diagrammatically in Figure 9. It must be emphasised that this suggested pleiotropy is only speculative. Other interpretations can be placed on the results, and other data may give different results. Tests of the conclusions drawn from this study would lie in obtaining data on hair growth-time relationships between genotype groups, and on mitotic rates in the follicle.

For the purpose of brevity, litter and sex effects on the variables studied have been left unexplained. The only variables for which significant sex effects were found were weaning weight and hair length, both of which showed reductions in females. Significant litter effects were apparent for most variables studied. This may indicate the sensitivity of hair characters to genetic backgrounds and/or maternal

DISCUSSION AND CONCLUSIONS

The results of the preliminary matings suggested that Mendelian segregations were occurring between dark and light mice, and that these might be due to the A/A vs A/a segregation at the agouti locus in the presence of one or more "umbrous" genes.

As an aid to the genetic investigation of the "dark" phenotype, objective measures of the dark phenotype were sought. As has been discussed earlier, comparisons of prepared skins from a litter gave no more accurate classification than that obtained from the live mice before skinning. This discrepancy with Mather & North's (1940) results may be due to the mice genotypes involved in the present study (d/+ A/a^t and +/+ A/a^t from Mating Mi.1) showing a smaller phenotypic difference than the genotypes studied by these authors. However, if the dark phenotype is reflected mainly in the length of the yellow agouti bands, which are subject to significant differences between litters, prepared "standard" skins would be of little use in classifying dark and light mice.

The most disappointing feature of the technique trials results was the difficulty in obtaining the measurements taken by Russell, E.S. (1946). This technique has been very powerful in the hands of its originator, in detecting the effects on hair pigment of alleles at the agouti, chinchilla, brown, pink-eye and dilute loci in both homozygous and heterozygous combinations with each other. As the procedure described by Russell, E.S.

(1946) was followed as closely as possible, it can only be concluded that the inability to measure the pigment variables resulted from lack of technical skill in slide preparation and measurement, and/or the use of a microscope which may have been sub-standard to that used by Russell.

The measuring of yellow band lengths and related variables on the projection microscope was easily carried out. The preliminary analysis of variance (Table 14) on yellow band lengths showed that this variable was reduced in dark ($Mi^{wh}/+,d/+,A/a$) mice as compared with light (normal $Mi^{wh}/+,A/A,A/a$) mice. This reduction in yellow band lengths of dorsal hairs of umbrous mice has been reported on at least two occasions (Mather & North 1940, Lane and Green 1960) although no objective results have been reported by these authors. Consequently mean yellow band length of mice, from the position sampled, was accepted as a satisfactory objective estimate of the dark phenotype for that mouse.

The results of the visual segregations provided evidence for the hypothesis that a single recessive "dark" gene was segregating, which has its phenotypic effect increased in A/a, as compared with A/A, mice. The genotypes A^{+}/a, A^{+}/a^{t}, A^{w}/a and A^{w}/a^{t}, all appear equally successful at increasing the phenotypic effect of both dark genotypes d/+ and d/d. The genotype-phenotype relationships on a visual basis appear to be:

+/+,A/a)
)
+/+,A/A) - light or "normal"
)
d/+,A/A)

d/+,A/a)
) - dark
d/d,A/A)

d/d,A/a - very dark

The same genotype-phenotype relationships hold for both $Mi^{wh}/+$ and +/+ genotypes although the $Mi^{wh}/+$ mice are lighter. The dark phenotype may be described as a "slight smudge" of dark colouration along the mid-dorsal line, while in the very dark phenotype this area is a colouration approaching black. Considerable variation within phenotypes occurs in the intensity of darkness. This phenomenon has been reported for other umbrous-like genes studied (Mather & North 1940, Robinson 1959), the latter author having pointed out the near polygenic nature of umbrous. The absence of clear segregations for Mating B.2 and the wild-type agouti mice of Matings Mi.1-5, is attributed to this overlapping or near-overlapping of phenotypes.

The results of the yellow band length studies supported the conclusions drawn from the results of the visual segregations. Dark mice had reduced mean yellow band lengths while very dark mice showed a further reduction. Classification on the basis of yellow band length adjusted for litter effects gave ratios of the phenotypes in agreement with expectation. However, overlapping values between phenotypes on the basis

of yellow band length occurred. Whether these "mis-classifications" compared with the visual classifications were due to sampling errors in the estimates of mean yellow band length, the overlapping between genotypes of true phenotypic values, or the estimates representing true phenotypic values of a continuous distribution of phenotypes, is not known. Consequently, using mean yellow band length as estimated from 8 zigzag hairs of a mouse, has not been a sufficiently accurate estimation of true phenotypic effect of the dark gene, to obtain evidence of a clear cut gene segregation.

The mutual reduction of dominance at umbrous and agouti loci has been reported for several authors who studied different umbrous genes (Barrows 1934, Mather & North 1940). The effect of the A/a genotype in this study was similar to that reported by Mather & North (1940) in that heterozygous non-agouti acted to darken the phenotype in both d/+ and d/d genotypes.

Evidence from the objective studies suggests that the dark genotypes reduce yellow band length from the basal end of the band (relative to the tip of the hair) and that this effect is independant of the $Mi^{wh}/+$ genotype's effect. It is a likely hypothesis that in Dominant White agouti mice, the sub-terminal black band would have been increased in length as in wild-type agoutis "had it been there". Markert & Silvers (1956) have suggested that the $Miwh$ allele has its effect in the cells creating the

environment for the melanoblast, such that the melanoblast environment is altered. Silvers & Russell (1955) and Silvers (1958) have shown that the agouti phenotype of the hair is also a consequence of the agouti genotype of the follicle. The absence of the black sub-terminal band in Dominant White agouti mice might thus reflect some interaction within the follicle cells of the Mi^{wh} and agouti alleles. The pleiotrophic studies indicate that in one dark genotype there is a direct effect of the gene on yellow band length, possibly on the yellow $\longrightarrow$ black switch mechanism at the basal end of the hair. Also the dark gene has an effect on yellow band length, acting through hair length, hence is likely that the three loci, Dominant White, Agouti and dark, act and interact in the hair follicle cells rather than in the melanocyte itself.

The dark gene studied here does not appear to resemble exactly any other umbrous gene described. A possibility of resemblance to the dark gene (da/da) reported by Falconer (1956, 1957) which arose in his C.B.A. inbred line, was dismissed on the basis that da/da mice showed reduced growth rates which were not evident in these studies.

The significant litter differences obtained in this study for many hair characters have been left unexplained. These effects on hair growth would make an excellent basis for a genetic study of both maternal environment and hair growth physiology.

The dark gene which is being stored in an inbred line based on

brother-sister matings in each generation ($d/d,A^w/a^t$ x $d/d,A^w/A^w$) is likely to be more useful for studies regarding the genes effect on hair growth than pigmentation studies.

Limitations of mice numbers used, together with the facts that only zigzag hairs were studied from one sampling position, no information was obtained on pigment characters, and the absence of objective measures of phenotype allowing clear-cut segregations to be observed, make the conclusions drawn from this study tentative only.

For purposes of brevity the phenotype described has been termed "dark"(d/d). This should not be taken to imply correspondence with the genotype da/da, or dilute (d/d).

SUMMARY

(i) An investigation of an apparently genetically controlled darkening of the mid-dorsal region of the agouti mouse coat is described.

(ii) Results from preliminary matings indicated that the dark effect was determined in a simple Mendelian manner and was modified by the genotype at the agouti locus.

(iii) Prepared matings were established to study the genetic character of the dark effect on both Dominant White and wild-type homo- and heterozygous backgrounds.

(iv) Results from these matings indicated that the dark effect was controlled by a single recessive gene, and that mutual modification of dominance occurred at the dark and agouti loci.

(v) Dark mice were shown to have shorter yellow agouti bands than normal mice, and consequently yellow band length was used as an objective measure of phenotype.

(vi) The results of the objective studies supported the conclusions drawn from the visual segregations.

(vii) A study was made of the pleiotropy of one dark genotype, the results of which are discussed in relation to interaction of the Dominant White, Agouti and Dark loci.

(viii) It was concluded that the dark gene closely resembles other modifiers of the agouti phenotype which are collectively termed "umbrous" genes, but does not appear to be one previously reported.

(ix) Reasons are given for only tentative conclusions being drawn from this study.

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APPENDIX I.

A glossary of conventional abbreviations used in the text.

N.S.	Results not statistically significant (at the 5% level in this study).
* or $p < .05$	Results statistically significant at the 5% leval.
** or $p < .01$	Results statistically significant at the 1% level.
F	The variance ratio
d.f.	Degrees of freedom
M.S.	Mean Square
s.s.	Sums of squares
R ( )	Reduction in sums of squares due to fitting the constants within the brackets
ssx^2	Sums of squares of x
ssxy	Sums of cross-products of x and y
ssy^2	Sums of squares of y
b	Regression coefficient
X^2_n	The chi-square statistic for n degrees of freedom
p or P	Probability
S.E	STANDARD ERROR OF A MEAN.

APPENDIX II

Descriptions of the inbred lines used in this study.

Line	Coat colour Phenotype	Genotype
C B A	Grey-bellied Agouti	A^{+}/A^{+}
$A^{w}.101$	Yellow-bellied Agouti	A^{w}/A^{w}
c_{57}	Black	a/a
C Y X *	Yellow-bellied grey	$Mi^{wh}/+$, $a^{t}/+$
	Grey	$Mi^{wh}/+$, a/a
	Yellow bellied black	+/+, $a^{t}/+$
	Black	+/+, a/a

* Not inbred for 20 generations