STUDIES ON SPOTTING PATTERNS. IV. PATTERN VARIATION AND ITS DEVELOPMENTAL SIGNIFICANCE

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Received June 5, 1938

INTRODUCTION

PERHAPS the most striking feature of the spotting patterns found in individuals or strains of many mammalian species is their great variety. They seem all to have the same immediate basis, failure of pigmentogenesis in the hair follicles of some portion of the skin. But the site of the failure is almost unique to each spotted individual.

Considerable variability persists even in strains where the chance of genetic segregation has been minimized by long inbreeding (DUNN 1920). For example, one strain reported in the first paper of this series (DUNN and CHARLES 1937) included individuals with few or no white hairs dorsally, others with unpigmented fur on as much as 25 percent of the dorsum, and a majority of intermediate grade. Among mice with about the same amount of white some had the unpigmented areas only on the left side, some only on the right and some on both.

That there is, however, a certain order in the intra-strain variation of spotting pattern has long been recognized (ALLEN 1914). Thus, in the strain cited above, no individual bore white hairs dorsally elsewhere than on the lumbar region and the distal portions of the legs and tail. Furthermore within the general lumbar area the central portion seems to be more frequently unpigmented than portions either more anterior or more posterior. Similarly there were more mice with white fur only on the feet and tail tip than with white fur extending more proximally on the legs and tail. Thus different body regions are much less sharply differentiated in their non-genetic variation than they are in the single spotted individual, as WRIGHT (1920) has pointed out. Each small skin area has a characteristic potentiality for developing pigment measurable by the proportion of animals within a strain bearing pigmented fur on the area; and the potentiality seems to vary systematically from area to area over the body surface, forming some sort of a gradient field which depends presumably on

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Columbia University. The writer is grateful to the Trustees and Council of Columbia University for a University Fellowship in Zoology (1933–4) during tenure of which part of the present work was done; and to Professor L. C. Dunn for many kindnesses during the course of the investigation.

systematic regional differences in the operating conditions of the relevant genes.

It is with the measurement and comparison of such gradient fields, in the presence of six different combinations of "spotting" genes, that the present study is concerned. It has seemed desirable at the same time to re-examine the pigmentogenic enzyme content of pigmented and colorless hair follicles previously studied by ONSLOW (1915) and BLOCH (1917) among others.

MEASUREMENT OF PATTERN VARIATION IN SPOTTED STRAINS AND HYBRIDS

Material

Four strains of mice from the colony of Professor L. C. DUNN, and three groups of hybrids among these strains, have been used. The origin and appearance of these strains, and the genetic basis of their spotting, have been described in detail in Part I (DUNN and CHARLES 1937).

Line K shows white fur on or near the lumbar region, generally in the form of a transverse white belt which may be partial or irregular or contain an "island" of white hairs. Line 190 is similar except that the white areas often seem to extend more posteriorly than any in Line K; there are more individuals with higher proportions of white fur. Line 66 ordinarily has very little white fur, in the form of a narrow unilateral belt or median spot in the lumbar region. Line 19 is "all white" except for a single rump or ear patch on a few individuals. If attention is confined to the proportion of white fur on the dorsal surface, without regard to its location, the four strains and the three groups of hybrids are as follows:

Percent of white on dorsum

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	N
	Percent of strain showing given amount of white																					
wild	100																					—
L66	18	73	7																			162
LK	1	3	18	20	24	27	7															262
L190			1		4	7	11	24	34	16	2											140
LK×L19					3	8	17	23	25	17												60
L66×L19									2	2	16	35	14	10	14	2	2		2	2		57
L190×L19										2	6	8	19	19	25	10	4	6				48
L19																			5	17	78	173

That the intra-strain pattern differences indicated in this table did not depend upon gene differences seems probable from the extent of previous inbreeding and from the mother-offspring, father-offspring correlation coefficients which are L66, 0.22, 0.22; LK, 0.16, 0.09; L190, 0.16, 0.

For convenience the four pure lines will sometimes be referred to in the following pages by their average proportions of white fur; that is, 4%

=L66; 20% = LK; 35% = L190; 100% = L19. The hybrids will be denoted by the product of the parent averages; for example (20%×100%) = LK×L19.

The 4% and 35% lines each carry the piebald gene "*s*" of the older literature. The 100% line has this gene and in addition at least three other mutant alleles tending to produce white spotting and acting cumulatively with *s*. The 20% strain has the wild type allele of *s* but otherwise carries most or all of the mutant alleles of the 100% line. The main ones of this latter group may be designated by *k*, *e*, *f*; it is probable that there are a few other mutant genes with minor effects in the 20% and 100% stocks. The tentative genotypes of the four strains are: 4% *ssKKEEFF*; 20%, *SSkkeeff*; 35%, *ssKKEEff*; 100%, *sskkeeff*, wild type being *SSKKEEFF*. For the present purposes the precise number of genes differentiating the strains is less important than the clear fact that the pattern differences among strains *are* genetically determined (DUNN and CHARLES 1937).

Method

Camera lucida tracings were made of the spotting patterns of the following mice, each at 10± days of age: 27 *ssKkEeff* (35%×100%); 31 *ssKkEeFf* (4%×100%); 21 *ssKKEEff* (35%); 48 *Sskkeeff* (20% ×100%); 94 *SSkkeeff* (20%); and 56 *ssKKEEFF* (4%). At the age when tracings were made the hairs are still quite short so that the superficial pattern corresponds closely with that at the surface of the skin.

Each mouse was etherized and so arranged beneath the camera lucida that its projected outline coincided as closely as possible with a standard diagram drawn on quadrille paper with tenth-inch rulings; the projected boundaries between colored and white areas were then traced on the diagram. (It was not always possible to make the two outlines coincide completely; in these cases the pattern boundaries were not traced exactly but were shortened or lengthened proportionately.) There were thus obtained a number of identical-area pattern records, identically subdivided.

Two representative tracings from each of the six genotypic groups are shown in figure 1. (In this figure the original quadrille rulings have not been reproduced and the areas bearing colored fur have been blacked in.)

Data

From the pattern tracings was obtained the "frequency of pigmentation" at each of 508 skin points for each of the six genotypic groups. This was done simply by counting what proportion of the tracings in a group showed pigmented fur at the center of the first square, what proportion at the center of the second square, and so on for each of the 508 squares into which the quadrille rulings divide the standard diagram.

The frequencies so obtained are represented in figures 2–4 where the height of each square pillar is the proportion of mice of the given genotype bearing pigmented fur at the center of the skin region beneath the pillar.

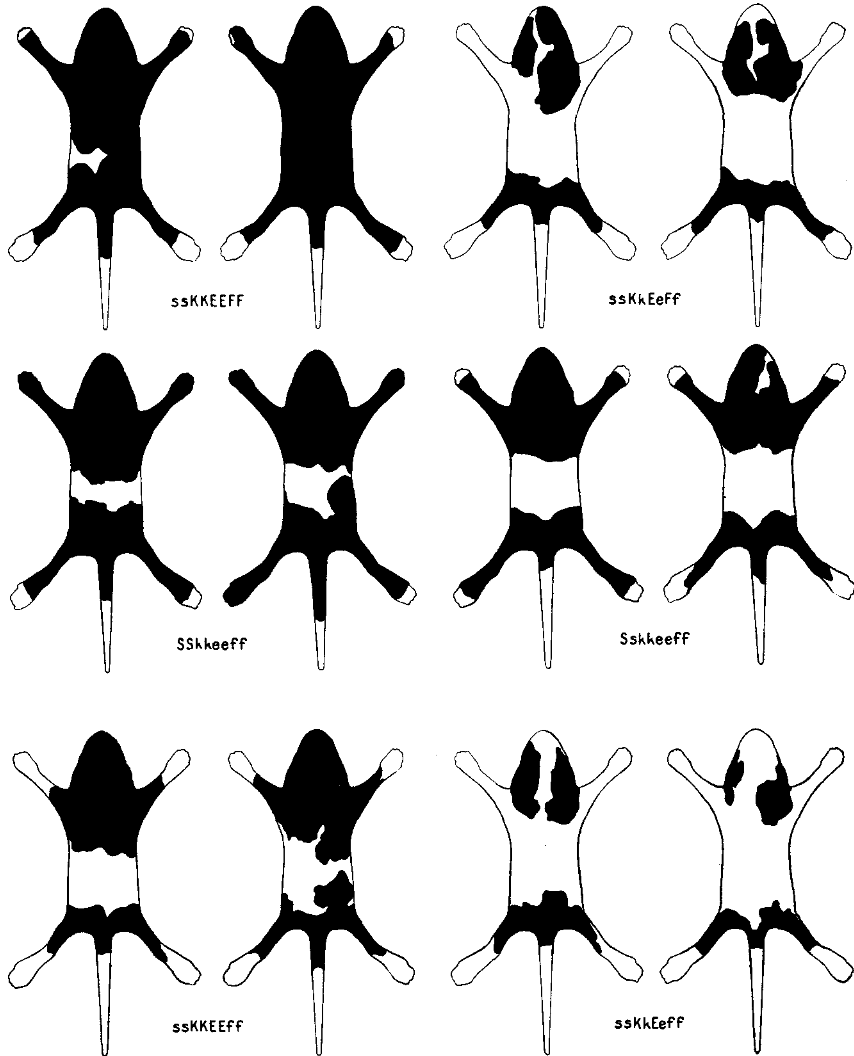


FIGURE 1.—Representative dorsal patterns of six genetic types of spotted mice.

Each figure is a gradient field measuring the regionally-variable potentiality for forming pigment in the presence of a particular gene combination.

The frequencies in figures 2–4 can be identified with "points" of skin only with certain qualifications arising as follows. In tracing the spotting

patterns, as has been indicated, it was not always possible to arrange the mouse to coincide exactly with the outlines of the diagram. In such cases

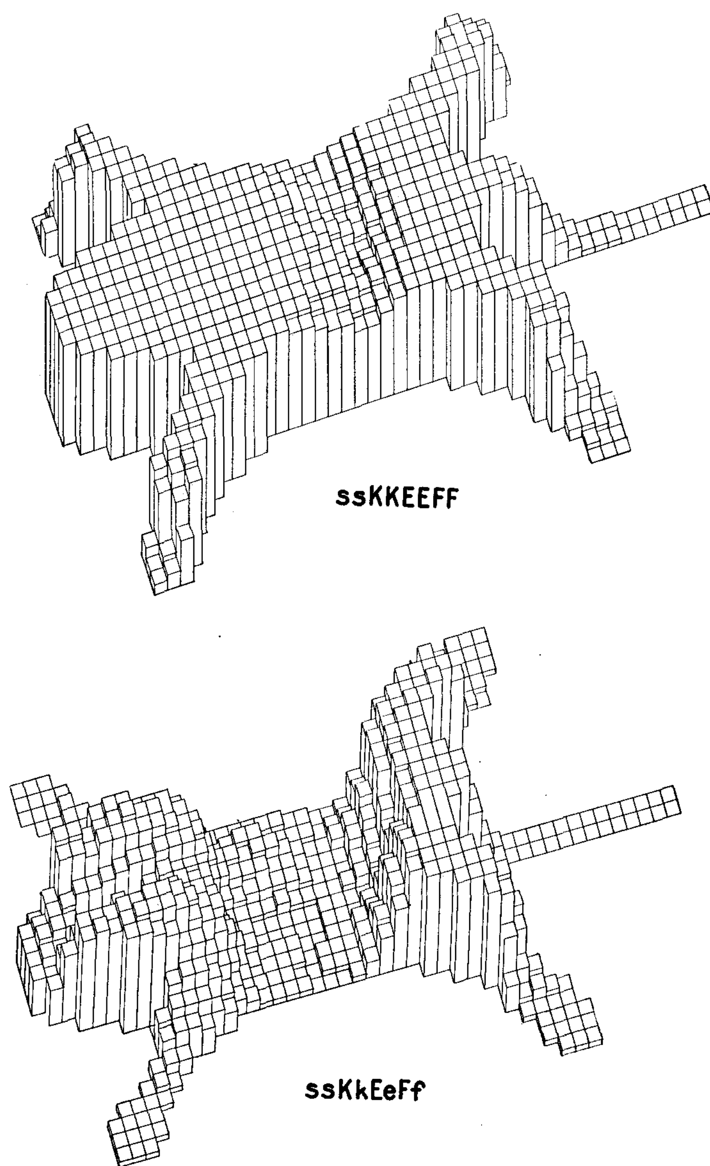


FIGURE 2.—Pigmentation frequencies at 508 skin points in *ssKKEEFF* and *ssKkEeFf* spotted mice.

it was necessary to enlarge or reduce portions of the projected pattern in the tracing process. In addition, slight changes in position of the eye during tracing tended to produce irregular shifts in position of the projected

pattern. In consequence even repeated tracings of the same mouse would be expected to show the same point of skin represented at somewhat different positions on the diagram. Conversely, a constant point of the diagram corresponds to somewhat different points on the different skins traced. The range of this uncertainty can scarcely have been less than 1 percent of the distance from nose tip to tail base, nor much more than 3 percent. Consequently the frequency of pigmented fur represented at a given point in a set of tracings is a complex average of the actual frequencies within an area of diameter equal to 1–3 percent of the body length. For convenience the frequencies will be referred to subsequently as though they described events at actual points of skin, but the limitation may be borne in mind.

The six gradient fields of figures 2–4 are represented as contour maps in figure 5. Each of these maps has been made by plotting on a standard diagram the series of points where the pigmentation frequency of one genotype is 10, 30, 50, 70 and 90 percent; in most cases the points were located by interpolation. The points were then connected by smooth lines, but they give a fair approximation to the contours of the three-dimensional figures.

It will be noticed that several of the maps have contour lines at very nearly the same location. The 30 and 70 percent lines on the head of *ssKkEeff* correspond very closely to the 50 and 90 percent lines, respectively, of *ssKkEeFf*. The 50 percent line on the anterior dorsum of *Sskkeeff* corresponds to the 90 percent line of *SSkkeeff*. Also in general the contour lines on each part of the body run in nearly the same direction for all genotypes, regardless of the actual values. Altogether it seems as though isopotential, that is similarly reacting, strips of tissue were being dealt with: whatever the pigmentation frequency of a particular point may be in the presence of a particular gene combination, all other points on the strip have very nearly the same value.

Two other general relations are readily seen by comparisons among the data of figures 2–4, as follows.

Relative height of frequency field in homozygote and heterozygote. Three such comparisons can be made: *ssKKEEFF* with *ssKkEeFf* (figure 2); *SSkkeeff* with *Sskkeeff* (figure 3) and *ssKKEEff* with *ssKkEeff* (figure 4). In each case the homozygote can be seen to have a higher pigmentation potentiality than the heterozygote at every point (except where both are 0 or 100 percent) as might be expected. That is, substitution of the wild type genes for the mutant alleles simply increases the pigmentation frequency at each point of the skin (except where the frequency is already 100 percent in the heterozygote). As to the magnitude of increment, the most obvious possibility is that it should be everywhere the same. That

such is not the case is shown by table 1: the increment varies with the pigmentation frequency in the heterozygote, and perhaps with the general

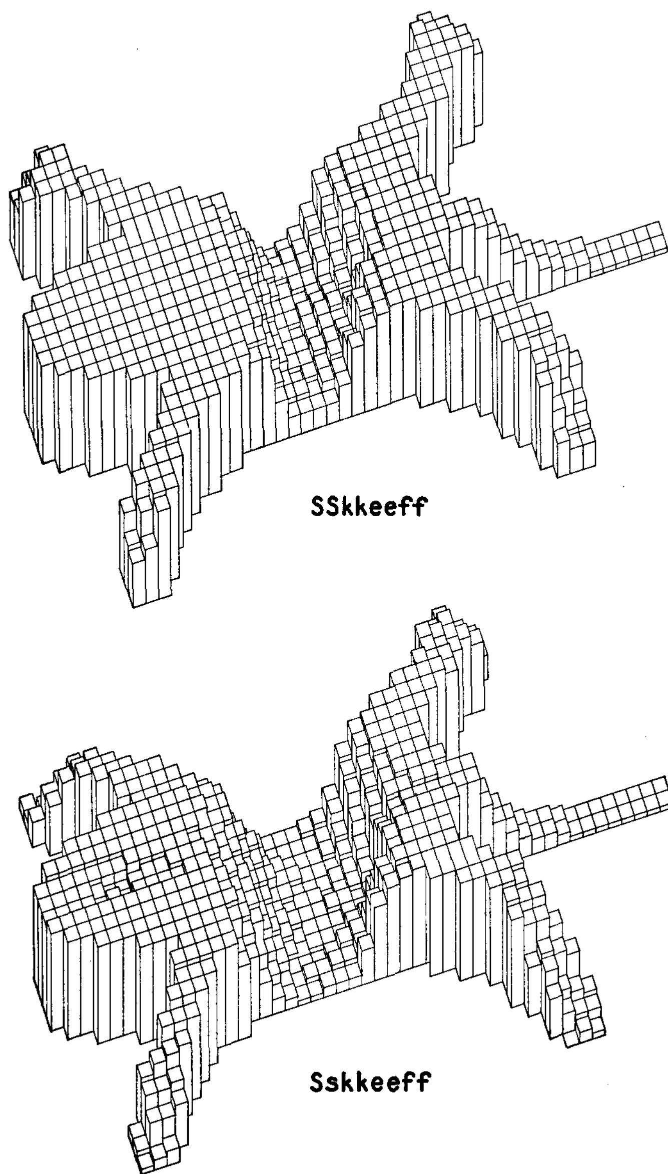


FIGURE 3.—Pigmentation frequencies at 508 skin points in *SSkkeeff* and *Sskkeeff* spotted mice.

region of the body. However, as will be seen in the analytical section, an approach to constancy of increment from heterozygote to homozygote is obtained if the pigmentation frequencies are transformed in a simple way.

Action of different loci on the frequency field. The simplest expectation here is that the six gradient fields should have the same general form and fall into a simple series with respect to height, their order being the same at every point of the skin. Such a situation might be expected if the *s*, *k*,

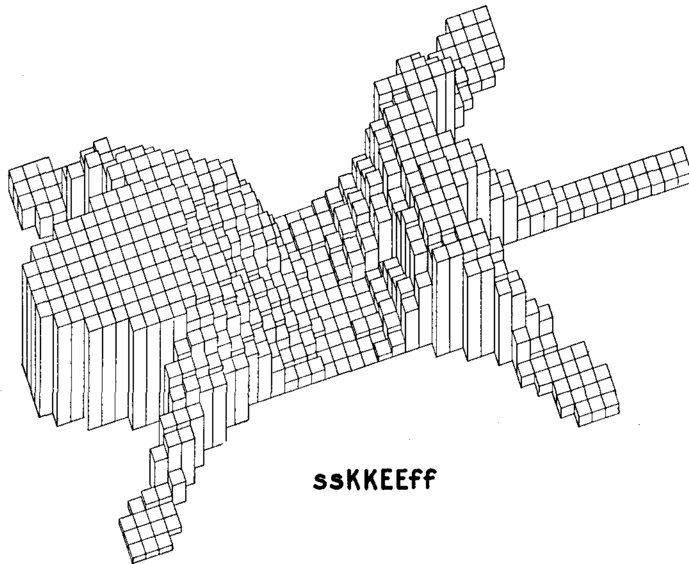
TABLE I

Pigmentation frequency, in percent, of corresponding skin points in heterozygous and homozygous spotted mice; the increments from heterozygote to homozygote are shown in brackets.

Sskkeeff (56 MICE)		AVERAGE FREQUENCY IN *SSkkeeff* (94 MICE)					
RANGE	AVERAGE	HIND LEGS AND TAIL	POSTERIOR DORSUM	ANTERIOR DORSUM	FORELEGS	HEAD	TOTAL POINTS
1–9	5	18 (13)	34 (29)	25 (20)	47 (42)	—	48
10–18	14	30 (16)	42 (28)	38 (24)	55 (41)	—	42
19–26	22	49 (27)	58 (36)	61 (39)	79 (57)	—	19
27–34	30	70 (40)	74 (44)	67 (37)	92 (62)	—	12
35–43	39	86 (47)	84 (45)	84 (45)	79 (40)	—	10
44–51	47	79 (32)	—	93 (46)	97 (50)	—	8
52–59	55	97 (42)	95 (40)	96 (41)	97 (42)	—	13
60–68	64	95 (31)	95 (31)	100 (36)	97 (33)	—	9
69–76	72	100 (28)	95 (23)	100 (28)	97 (25)	—	17
77–84	80	100 (20)	98 (18)	100 (20)	98 (18)	—	29
85–93	89	100 (11)	98 (9)	100 (11)	100 (11)	—	17
94–99	96	98 (2)	100 (4)	100 (4)	100 (4)	—	46
100	—	100	—	100	100	—	79
ssKkEeff (27 MICE)		AVERAGE FREQUENCY IN *ssKKEEff* (21 MICE)					
0	—	9	4	27	41	—	137
1–9	5	9 (4)	6 (1)	48 (43)	42 (37)	—	98
10–17	13	33 (20)	32 (19)	73 (60)	64 (51)	96 (83)	34
18–24	21	36 (15)	40 (19)	76 (55)	74 (53)	94 (73)	23
25–32	29	—	59 (30)	85 (56)	—	100 (71)	12
33–39	36	—	83 (47)	89 (53)	—	100 (64)	15
40–47	44	55 (11)	78 (34)	93 (49)	—	99 (55)	28
48–54	51	—	77 (26)	93 (42)	—	100 (49)	14
55–62	58	51 (−7)	95 (37)	95 (37)	—	100 (42)	17
63–69	66	71 (5)	—	100 (34)	—	100 (34)	10
70–77	73	82 (9)	92 (19)	—	—	100 (27)	15
78–84	81	96 (15)	94 (13)	—	—	100 (19)	23
85–91	88	97 (9)	100 (12)	—	—	100 (12)	20
92–99	95	98 (3)	100 (5)	—	—	100 (5)	18
100		—	100	100	—	100	20

e and *f* alleles all had the same sort of primary effect, but different degrees of activity, as WRIGHT (1920) has assumed to be the case for a group of spotting genes in the guinea pig. The expectation seems to be fulfilled in the case of *ssKKEEFF* (4%), *ssKKEEff* (35%), *ssKkEeFf* (4%×100%) and *ssKkEeff* (35%×100%). Six comparisons can be made among these genotypes, taking two at a time: *ssKKEEFF* and *ssKKEEff* have already

been seen to be consistently higher than *ssKkEeFf* and *ssKkEeff*, respectively; direct comparison of figures 2 and 4 shows that *ssKKEEFF* is



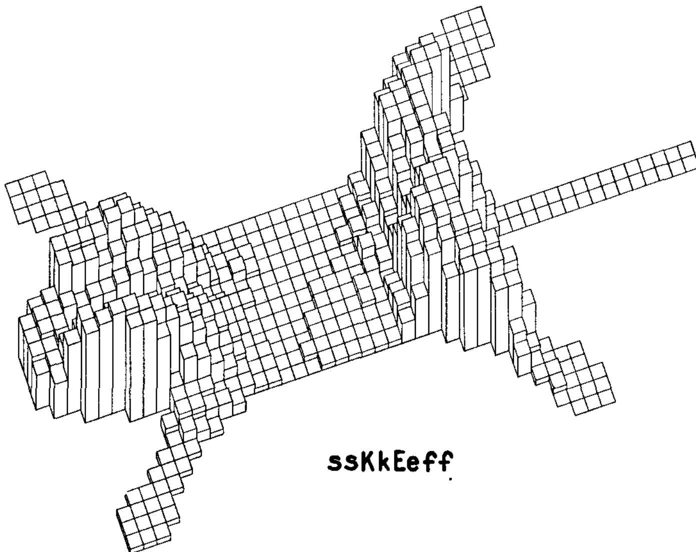


FIGURE 4.—Pigmentation frequencies at 508 skin points in *ssKKEEff* and *ssKkEeff* spotted mice.

higher than *ssKkEeff*, *ssKKEEff* higher than *ssKkEeFf*. Two comparisons remain to be made: *ssKKEEFF* (4%) with *ssKKEEff* (35%) and *ssKkEeFf* (4%×100%) with *ssKkEeff* (35%×100%); these are shown

in table 2. The *ssKKEEFF* field is higher than that of *ssKKEEff*, or equal to it at 100 percent, except at 42 of the 508 points. Nearly all of these points are on the extreme distal portions of the hind legs and tail where the data are least reliable since only few animals of either genotype bear pigmented fur there.

The same sort of relation is found between the frequency fields of *ssKkEeFf* and *ssKkEeff*. The former is equal to, or greater than, the latter at 431 of the 495 points recorded in table 2. Of the remaining 64 points, 48 bore pigmented fur in none, or only one, of the 31 *ssKkEeFf* mice and in one, or two, of the 27 *ssKkEeff* mice; the other 16 points had very nearly

TABLE 2

Relative frequency of pigmentation at corresponding skin points of different spotting genotypes.

RELATIVE FREQUENCY	NUMBER OF POINTS					
	HIND LEGS AND TAIL	POSTERIOR DORSUM	ANTERIOR DORSUM	FORE-LEGS	HEAD	TOTAL POINTS
ssKKEEFF>*ssKKEEff*	55	86	117	59	6	323
ssKKEEFF<*ssKKEEff*	39	0	0	3	0	42
ssKKEEFF=*ssKKEEff* =100%	24	28	3	0	88	147
ssKkEeFf>*ssKkEeff*	83	80	95	34	80	372
ssKkEeFf<*ssKkEeff*	2	20	11	17	14	64
ssKkEeFf=*ssKkEeff* =0% or 100%	30	11	7	11	0	59
ssKKEEff>*Sskkeeff*	22	8	44	2	29	105
ssKKEEff<*Sskkeeff*	68	80	73	50	6	277
ssKKEEff=*Sskkeeff* =100%	24	26	3	0	59	112

equal pigmentation frequencies. Since the differences at these discordant points are so small, they may perhaps be only a consequence of sampling error.

Thus it seems probable that the frequency fields of *ssKKEEFF*, *ssKKEEff*, *ssKkEeFf* and *ssKkEeff* fall into a consistently diminishing series in the order indicated and that the *KE* and *F* wild type genes may thus have the same sort of primary or intermediate action.

There seems however to be a different relation between the genotypes containing *KE* without *S* and those containing *S* without *KE*. The most extensive comparison available is between *ssKKEEff* (35%) and *Sskkeeff* (20%×100%), in table 2. The significant regions here are the head, where *Sskkeeff* often shows a white blaze although *ssKKEEff* never does,

and the posterior dorsum, where *Sskkeeff* has consistently higher pigmentation frequencies. A similar reversal in relative height of fields can be seen by comparing figures 2 and 3; over the general lumbar area, *ssKKEEFF* has much higher pigmentation frequencies than *SSkkeeff*; but on the distal halves of the legs *SSkkeeff* is higher. It seems very unlikely, from the sizes of the two samples and from the range of frequencies over which the reversal is observed, that the reversal is only a matter of sampling error.

SSkkeeff (20%) and *Sskkeeff* (20%×100%) thus do not fit into a consistent series with the other four genotypes, but have a pigmentation frequency field of fundamentally different shape. It seems probable accordingly that *S* has a different primary or intermediate action than *K*, *E* and *F*.

Correlations in pattern variation. A fourth general phenomenon of the variation in spotting pattern is the interrelation in pigmentation response (that is, forming or not forming pigment) among large groups of skin points. This has been previously studied by ALLEN (1914) in genetically heterogeneous groups from various mammalian species and by ILJIN (1928) in guinea pigs. The immediately observable fact is that the skin of any spotted animal (except in the case of dominant spotting) consists of a small number of large pigmented or unpigmented regions. Furthermore there is a limited variation in the arrangement of the two kinds of regions, within any homogeneous stock. In the lighter genotypes (*ssKkEeFf*, *ssKkEeff*) there are typically three patches of colored fur dorsally: one covering both sides of the rump and extending somewhat onto the legs and tail; one centering on each ear and extending towards the eyes and scapula. The two ear patches may be confluent; there may be an "island" of colored fur somewhere between the ear and rump patches. In the darker genotypes (*ssKKEEFF*, *SSkkeeff*) there are rarely more than six regions of white fur, dorsally; one on each foot, one on the tail tip and one on the lumbar area. The foot and tail patches may extend for various distances centrally; there are occasionally two lumbar patches; in *SSkkeeff* there is sometimes a white blaze on the forehead. In all groups no part of the leg or tail is pigmented unless there are also pigmented hairs on all more proximal parts of the appendage at least as far as the point indicated by the intersection of the dotted lines from leg and tail in the *ssKKEEFF* contour map in figure 5. Conversely if the knee, or tail base, bears *white* hairs, all more distal parts of the appendage do also. No part of the foreleg is pigmented unless there are pigmented hairs as far centrally as the base of the ear (intersection of the dotted lines on arm and body proper in figure 5). The anterior tip of the head is never pigmented unless there is colored fur at least as far posteriorly as the rear of the eye.

Although leg and tail pigmentation seem thus to depend on the formation of pigment more centrally they do not depend on each other. That is (in *SSkkeeff*, *Sskkeeff* and *ssKKEEFF*) the proportion of animals with pigment at any one point on the leg and any one point on the tail has not

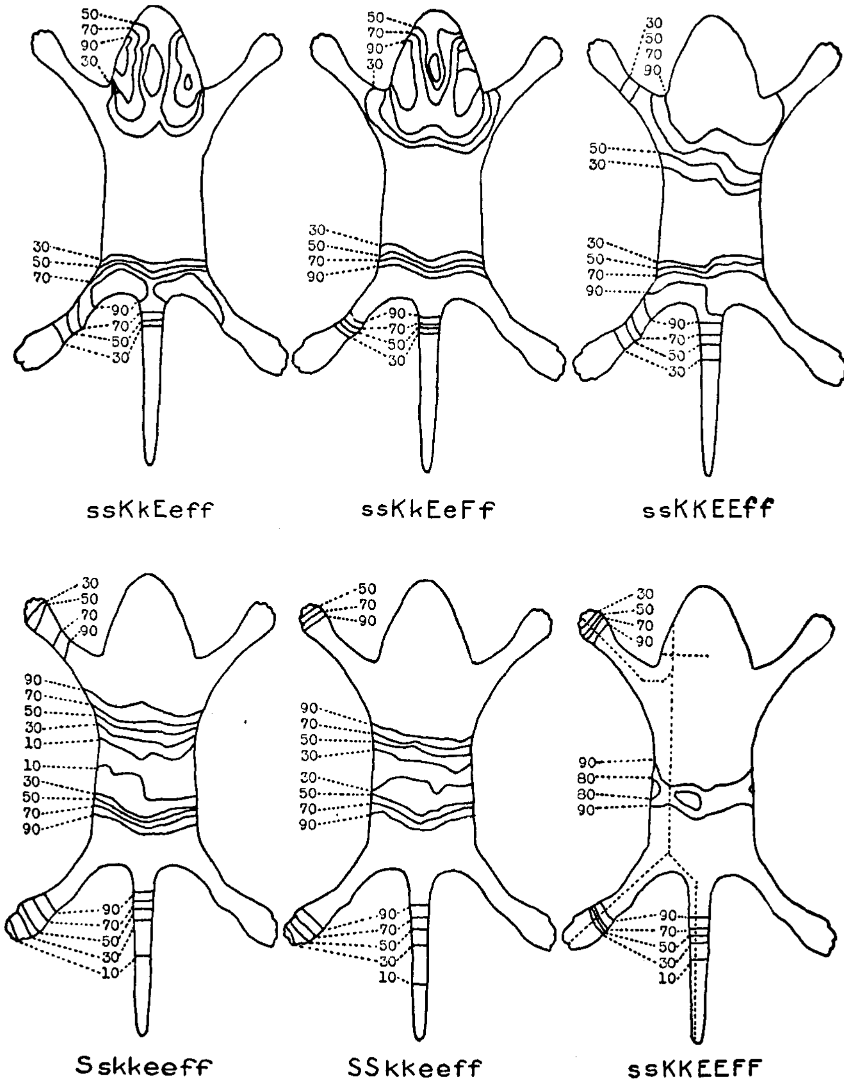


FIGURE 5.—Contours of the pigmentation frequency surfaces in figures 2–4.

been found to be significantly different from the chance expectation, that is, from the product of the pigmentation frequencies of the two points. Similarly no relation has been found between pigmentation of points on the tail and foreleg, on hind and fore leg or on opposite sides of the body.

A somewhat different situation is found in the central two-thirds of the body proper, that is, the central two-thirds of the longitudinal dotted line on the *ssKKEEFF* contour map in figure 5. The coefficients of association in pigmentation at pairs among ten points along this axis are shown in table 3, for *SSkkeeff* which is the only group of tracings large enough to give fairly reliable results. There is a consistent association in presence or absence of pigment between any pair of adjacent points and

TABLE 3

Association in presence or absence of pigment at ten equidistant points extending from shoulder to rump (along the dotted line in figure 5) in SSkkeeff spotted mice. N_{ij} *is the number of animals with pigment at each of two points;* N_i *with pigment at the more anterior point only;* N_j*, at the most posterior point only;* N_0*, at neither; c is the coefficient of association,* $(N_{ij}\cdot N_0 - N_i\cdot N_j)/(N_{ij}\cdot N_0 + N_i\cdot N_j)$.

POINTS	N_{ij}	N_i	N_j	N_0	c	POINTS	N_{ij}	N_i	N_j	N_0	c
1.2	52	30	0	12	1	3.9	17	6	59	12	−0.27
1.3	23	59	0	12	1	3.10	21	2	69	2	−0.53
1.4	21	61	2	10	0.27	4.5	19	4	5	66	0.97
1.5	23	59	1	11	0.62	4.6	17	6	14	57	0.84
1.6	28	54	3	9	0.22	4.7	16	7	22	49	0.67
1.7	31	51	7	5	−0.39	4.8	17	6	33	38	0.53
1.8	41	41	9	3	−0.50	4.9	17	6	59	12	−0.27
1.9	65	17	11	1	−0.48	4.10	20	3	70	1	−0.83
1.10	78	4	12	0	−1	5.6	21	3	10	60	0.95
2.3	21	31	2	40	0.86	5.7	19	5	19	51	0.82
2.4	14	38	9	33	0.15	5.8	20	4	30	40	0.74
2.5	14	38	10	32	0.08	5.9	20	4	56	14	0.11
2.6	17	35	14	28	−0.01	5.10	21	3	69	1	−0.82
2.7	17	35	21	21	−0.35	6.7	28	3	10	53	0.96
2.8	24	38	26	16	−0.31	6.8	28	3	22	41	0.94
2.9	40	12	36	6	−0.29	6.9	27	4	49	14	0.32
2.10	50	2	40	2	0.11	6.10	27	4	63	0	−1
3.4	16	7	7	64	0.91	7.8	32	6	18	38	0.84
3.5	12	11	12	59	0.69	7.9	36	2	40	16	0.74
3.6	13	10	18	53	0.59	7.10	36	2	54	2	−0.20
3.7	12	11	26	45	0.31	8.9	43	3	29	15	0.78
3.8	15	8	35	36	0.31	9.10	74	2	16	2	0.64

a decreasing association between points successively further apart. From each point the association falls to zero and then assumes negative values.

The meaning of these negative values is not clear, but they must arise in part from the tracing method. The inaccuracies of alignment of the projected outline of a mouse against a standard diagram are such that, if a given mouse were traced repeatedly, the boundaries of a given white spot would not lie identically on the tracing. In general where the anterior boundary of the spot has been traced too far forward, the posterior boundary will also be too anterior; where the front boundary has been traced

further back, the rear boundary will also be relatively posterior. In repeated tracings of the same mouse there should thus be an association coefficient of −1 between presence of pigment at one point and another separated from it by the length of the spot traced. In tracings of different mice there will be a tendency to similar negative association between any two points which may lie immediately fore and aft of a white spot. Because of this error it is difficult to attach any meaning to the negative coefficients in table 3; the positive values indicate a real association.

In general, the formation of pigment on either the posterior or anterior dorsum shows a decreasing association with pigment on points successively nearer the opposite end of the body. Formation of pigment in the mid-dorsum is decreasingly associated with the pigmentation response of points successively further away either anteriorly or posteriorly. It seems probable that a similar relation holds for all six genotypes.

ANALYTICAL

Pigmentation frequencies. WRIGHT (1920, 1936) has used an "inverse probability transformation" to convert the proportion of white fur in the skin of a spotted guinea pig into a quantity which is equivalent to an average "concentration" of some material throughout the skin, relative to a "critical concentration" for pigment formation. The same transformation may be used to convert the pigmentation frequency of one skin point within a genotype into an "average concentration" at that point. The basis of the transformation is as follows.

"Although the skin of a piebald guinea pig is divided sharply into areas in which pigment is either produced to the full amount characteristic of the animal, or is wholly absent, it is not to be supposed that the influences, which at some critical period in ontogeny determine whether a region is to be colored or white, are so sharply alternative in themselves. It seems more reasonable to suppose that the sums of favorable and unfavorable influences in different parts of the skin could be arranged in a graded series. Doubtless in certain white regions a slight difference in the conditions would have enabled color to develop, while in others a great change would have been necessary. Similarly with colored areas. Suppose, then, that the skin is divided into a large number of equal areas and that it were possible to determine the true potentiality of each area at the critical period in development. . . . All areas which exceed this a 'critical potential' produce color, while those which fall below, however slightly, remain white." (WRIGHT 1920.)

The "true potentiality" of the cells ancestral to a small area *of a particular skin* at the "critical period in ontogeny" might be thought of, in the simplest possible way, as a concentration P of some unknown material.

The "critical potential" would then be the smallest concentration P_0 which could bring about the ultimate formation of pigment. Pigmented hairs would be borne on an area only if P had been equal to, or greater than, P_0. Since some regions of an individual bear pigmented fur, others white fur, either P or P_0 or both must vary from region to region of the same skin. Since the same small skin area may have pigmented fur in some individuals and white fur in other individuals of the same genotype, either P or P_0, or both, of that area must vary somewhat from animal to animal, P being the greater in some, P_0 in others. Since the spotting patterns are at least roughly similar among animals of the same genotype, the regional variation in P or P_0 must have the same general form throughout the group; that is, both P and P_0 must tend to a modal value for each skin

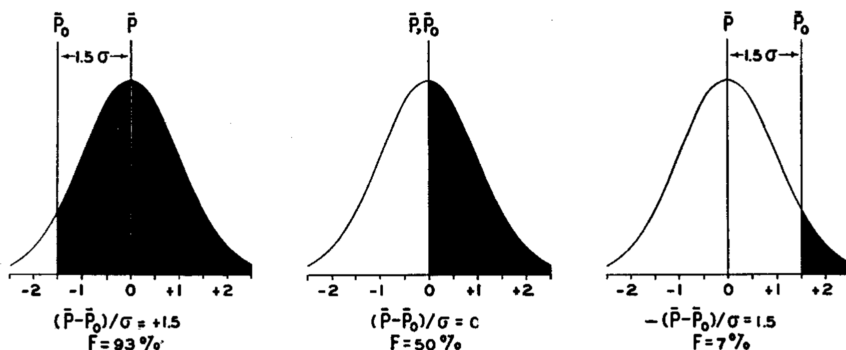


FIGURE 6.—Relation of pigmentation frequency (F, shaded area) at a skin point to average difference between a variable "concentration" P at that point and a minimal "concentration" P_0 for pigment formation (modified from Wright 1920).

point among individuals of the same genotype. Let us assume that the frequencies of various values of the difference $(P-P_0)$ for a given small skin area would form a normal distribution of standard deviation σ; the average value of the difference may be represented by $(\overline{P}-\overline{P}_0)$. The pigmentation frequency F to be expected for any value of $(\overline{P}-\overline{P}_0)$, relative to σ, can be found from a table of areas under the normal curve. That is, F is the probability function of $(\overline{P}-\overline{P}_0)/\sigma$. Three examples are shown in figure 6.

Conversely, the value of $(\overline{P}-\overline{P}_0)/\sigma$ corresponding to a particular F can be found from a table of the normal curve areas; that is, $(\overline{P}-\overline{P}_0)/\sigma$ is the inverse probability function of F. A few pairs of values are as follows:

F (in %) =	1	2	5	10	20	50	80	90	95	98	99
$(\overline{P}-\overline{P}_0)/\sigma$ =	−2.32	−2.06	−1.64	−1.28	−0.84	0	+0.84	+1.28	+1.64	+2.06	+2.32

This inverse probability transformation might be applied to each of the values in figures 2–4. No additional information would be gained, though: the relative steepness of various parts of the surfaces would be changed, but the order of their height, and the regional reversal of height between

Skef and *sKEF* genotypes, would not be altered. Insofar, however, as $(\overline{P}-\overline{P}_0)/\sigma$ may be thought of as a quantity like a difference of two concentrations, it is closer than a pigmentation frequency to the sort of variable on which gene substitution might be expected to have an equal additive effect throughout the skin. If this were so, the $(\overline{P}-\overline{P}_0)/\sigma$ values

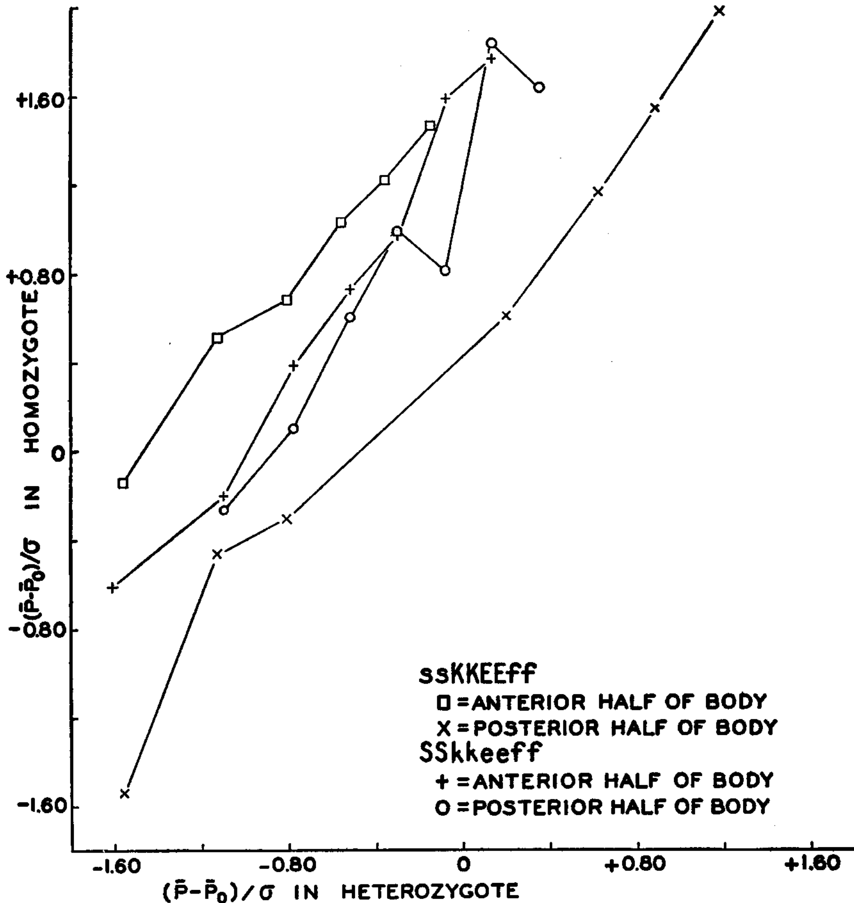


FIGURE 7.—Relation between transformed pigmentation frequencies of a heterozygous spotted type and the corresponding homozygote.

for a series of points in the homozygote, plotted against the values for the same points in the corresponding heterozygote, ought to fall along a straight line of slope 1. The transformed data of table 1 have been plotted in this way in figure 7 (the values for forelegs and anterior dorsum have been grouped together, as have the values for hind legs and posterior dorsum).

Three general relations seem to be indicated by figure 7:

(1) Within either the anterior or posterior half of the body the $(\overline{P}-\overline{P}_0)/\sigma$

values of *ssKKEEff* (35%) do fall along a straight line of slope 1 against the corresponding values in *ssKkEeff* (35% × 100%). That is, there is a constant increment from heterozygote to homozygote as though *K* and *E* had a simply additive effect; but

(2) The constant increment within the anterior half of the body is much greater than the increment within the posterior half; the values are approximately 1.6 and 0.6. (There is some indication of an even higher increment on the head proper than on the forelegs and anterior dorsum.)

(3) The $(\overline{P}-\overline{P}_0)/\sigma$ values of *SSkkeeff* (20%) fall along approximately the same straight line against the *Sskkeeff* (20% × 100%) values for both halves of the body, but the line seems to have a slope greater than 1. This is as though *S* had some sort of multiplicative effect. Here again a difference in nature of primary action between *S* and *KE* is indicated.

Correlations. From a comparative study of spotted mammals and birds ALLEN (1914) concluded that in both classes the skin consists dorsally of eleven contiguous or slightly overlapping cell groups within each of which, at some stage of development, a substance or process necessary for subsequent pigment formation spreads from the center towards the edges; that the extension occurs independently in the different areas and to its full extent in animals which become unspotted; that where the extending process stops short of the edge of its area, the peripheral territory becomes unpigmented; and that the spotting pattern of an individual is identical with the extent of spread of the determining process from the centers of the eye, ear, scapular, lumbar and rump areas of each half of the body.

ILJIN (1928) reached a different conclusion primarily from correlation studies on spotted guinea pigs: that spotting patterns are determined, not by restriction of a process which occurs in unspotted animals, but by extension of an action found only in spotted individuals; that in the mouse this action spreads from each of twelve "points of depigmentation" on the dorsal surface; and that between some of these points there is a correlation in degree of extension, the magnitude of correlation depending on the distance between the points considered.

ILJIN's "points of origin of depigmentation" coincide very closely with the margins of, or boundaries between, ALLEN's pigmentation areas. Both are located, in the main, at the common low points of the present pigmentation frequency fields, that is on the toes and tail tip, nose tip, center of forehead and anterior lumbar region.

ILJIN assumed depigmentation centers at front and rear of the third-quarter of the dorsum; ALLEN, a pigmentation center in about the middle of this quadrant. Now let two points be considered, one near the front, one near the rear of the third quadrant. If both points bear unpigmented fur in a given mouse it is indicated, according to ILJIN, that one has not

been reached by a process spreading from the anterior center, the other by a process from the posterior center. According to ALLEN, neither has been reached by one process diffusing from a single center. Now this quadrant of the dorsum corresponds to the series of points from 5 through 10 in table 3 among which most pairs of points show a reasonably large pigmentation association. These associations would mean peripheral correlation within one spreading process according to ALLEN, correlation between two processes under ILJIN's scheme. The former is at least simpler, though not necessarily more probable; it is equivalent to supposing that any one extending process tends to spread nearly equally in all directions; that the variation in extent is like the variation in aperture of an iris diaphragm rather than like an irregular, amoeboid variation in outline.

But ALLEN's scheme meets a certain amount of difficulty in the data of table 3. Point 9 has been shown never to be pigmented without pigmented fur extending from there to the posterior margin of the rump, which would mean that the last quarter of the dorsum lies in the rump field. But the pigmentation response of point 9 is positively associated also with that of points as far forward as the center of the dorsum (point 5), which would seem to mean that points 5 and 9 lie in ALLEN's lumbar field. The same difficulty is met at point 2, which seems to lie in both scapular and lumbar fields.

One possible solution is suggested by those skins which show "islands" or "peninsulas" of pigmented fur surrounded by colorless fur (figure 1). Under ALLEN's hypothesis these are cases where the spreading processes of three adjacent fields have failed to meet at their two borders. They may be found in three general positions centering approximately at points 1, 5 and 9 of table 3; that is, at the anterior end of the second quadrant, between second and third quadrants, and at the posterior end of the third quadrant. The three types of island are not sharply demarcated, but they do have a modal tendency. The first and third correspond to restriction of ALLEN's shoulder and lumbar fields, respectively. But the second is at the boundary between these two fields, and so might be supposed to define another pigmentation center ("mid-dorsal") not indicated by ALLEN, covering part of the territory of his scapular and lumbar areas. Within this common region, it might be supposed that pigmentation is sometimes determined by the spreading process from one center, sometimes by that of the other center, or perhaps by both jointly. One further alteration in ALLEN's scheme would be necessary from the present data. Since pigmentation on the foreleg does not occur without pigment on the ear, the former must belong to the ear field, rather than to the scapular as ALLEN supposed. Thus, if overlapping local fields are involved at some stage in the

embryogeny of spotting pattern, they perhaps number six on each side of the body: eye, ear, scapular, mid-dorsal, lumbar and rump.

In the absence of any direct demonstration the pigmentation fields of ALLEN and ILJIN, inferred from the regularities in location of pigmented and white fur areas, and the "concentration gradients," inferred from the regional variation in pigmentation frequency, are both merely ways of restating the primary data of genetic and non-genetic variation in spotting patterns. Each suggests that the next step in the analysis of the developmental basis of spotting patterns is a search for conditions which vary systematically thoughout the embryonic skin (and also to a certain extent from individual to individual), for example, time of attaining some particular stage of morphological differentiation, rate of growth, etc. Some such pattern seems almost certainly involved, determining that in some regions of the skin cells containing several mutant (perhaps inactive) genes can form pigment whereas in other regions of the skin the whole wild-type gene set must be present. Regarded in this way the formation of mammalian spotting patterns is a relatively superficial and accessible analogue to the more fundamental ontogenetic processes by which genes and cytoplasmic differentials, tracing back ultimately to the structure of the fertilized egg, together determine the location of morphological elements more important than hair pigment. At least from this aspect they deserve further study.

THE PHYSIOLOGICAL DIFFERENCE BETWEEN COLORED AND WHITE AREAS

The question has frequently arisen what component of the reaction system leading to presence of pigment varies in the skin of a spotted mammal. ONSLOW (1915) and BLOCH (1927) each reported the white-furred skin regions of spotted mammals to lack a melanogenic enzyme which is present in the colored-furred areas and throughout the skin of unspotted animals. ONSLOW identified the enzyme as tyrosinase (rabbits, mice); BLOCH, as a more specific "dopa oxidase" (guinea pigs). The latter has since come to be rather generally accepted as the melanogenic enzyme of mammals.

PUGH (1933) reported that tyrosinase can only seldom be found even in extracts of all-black skin. In about 15 experiments she found no evidence of tyrosinase activity even when ONSLOW's procedure was followed as closely as possible. But in a further set of five extractions, two did produce a grey coloration in tyrosine solution after 30–33 hours. She concluded that black rabbit skin does contain a true tyrosinase.

BLOCH (1917, 1927) reported that frozen sections of skin, immersed in a 0.1% solution of dioxyphenylalanine pH 7.3–7.4, in 6–12 hours show formation of additional pigment granules *only* in those places where

melanin had previously formed naturally—in the melanoblasts. Among a number of mono- and di-hydric phenols only dioxyphenylalanine gave the reaction. On this basis BLOCH concluded that mammals, unlike great numbers of invertebrate and plant species, do not contain a tyrosinase but have another pigment forming enzyme which is substrate specific to dioxyphenylalanine.

BLOCH found the dopa-oxidase reaction of sections of white-spotted guinea pig skin to be negative in the follicles producing white hairs and positive in those forming pigmented hairs. But PRZIBRAM, DEMBOWSKY and BRECHER (1921) have suggested, from evidence that regions in active melanogenesis are more alkaline than the surrounding tissue, that the dopa reaction is identical with the spontaneous oxidation which dopa undergoes in vitro at alkalinities not much great than pH 7.5.

Hence so far as can be told from the published material, there exists an anomalous situation with respect to the physiological differences between colorless and pigment-forming follicles of spotted mammals: an enzyme difference is reported by each of two experimental methods, but doubt is cast on the validity of each. If PUGH'S finding is correct that even with ONSLOW'S extraction method only about two out of seventeen samples from black skin show tyrosinase activity, then ONSLOW'S results may have been accidental; if (as is not clear from his report) he tested only one or two white skin extracts, the failure of tyrosinase activity in these cases may have been only an instance of the same random failure which PUGH observed even in black extracts. And BLOCH'S report of a dopa-oxidase difference between colored and unpigmented follicles in frozen sections may have been non-enzymic depending only upon the reported greater alkalinity of the pigment-producing follicles.

Accordingly it has seemed desirable to re-examine the existence of an enzyme difference between colored- and white-furred areas of piebald mouse skin. Because regular results could not be secured in preliminary tests with skin extracts and dopa, tyrosinase was used as a substrate in further study which was carried out as follows.

Comparable tests were made of the tyrosinase activity of extracts of skin of black unspotted mice and of extreme spotted, "all white," mice (DUNN and CHARLES 1937) only few of which have any pigmented hairs. Groups of 8–12 animals 2–4 days old were decapitated and bled as thoroughly as possible. The skins were removed with minimal connective tissue adhering and ground in a glass mortar with sand, chloroform, water or Ringer solution and pH 7.4 phosphate buffer (the amounts are shown in table 4). The resulting fairly homogeneous, gelatinous, grey or pale pink paste was centrifuged and filtered through a Buechner funnel. The opalescent filtrate was divided among a number of test tubes to which were

TABLE 4

Preparation of skin extracts for tyrosinase tests.

EXTRACT NUMBER	COLOR OF SKIN	NUMBER OF SKINS	EXTRACTION IN			
			pH 8 BUFFER	WATER	RINGER	$CHCl_3$
I.	black	8	1.5 cc.	—	3.5 cc.	0.5 cc.
II.	albino	8	1.5 cc.	—	3.5 cc.	0.5 cc.
III.	black	11	1.5 cc.	—	3.5 cc.	0.5 cc.
IV.	black	10	1.5 cc.	—	3.5 cc.	0.5 cc.
V.	black	12	5.0 cc.	—	—	5.0 cc.
VI.	white*	12	5.0 cc.	—	—	5.0 cc.
VII.	black	9	2.0 cc.	3.0 cc.	—	few drops
VIII.	white*	9	2.0 cc.	3.0 cc.	—	few drops

* "All white" extreme spotted.

added substrate as shown in table 5 and a few drops of chloroform. Tubes were kept either at room temperature or at 38°C. as indicated, and observed after intervals varying from 12–24 hours. The results are shown in table 5.

In the first series tyrosinase activity, without peroxide and so meeting the criteria for a true tyrosinase, was found in the extracts of two out of three groups of black skins (I and III). The degree of melanization after 20

TABLE 5

Tyrosinase activity of skin extracts.

EXTRACT NUMBER	COLOR	TEMP. (°C.)	TIME (HRS.)	1 CC. H^2O	1 CC. DOPA 0.04%	1 CC. TYRAMINE 2.02%	1 CC. TYROSINE 0.04%	
H^2O	—	20±	17	—	++	—	—	
I.	black	20±	17	—	+++*	—	+++	
II.	albino	20±	48	—	+++	—	—	
III.	black	38	19	—	+++	—	+++	
III. (diluted 1:5 with water)	black	38	19	—	+++	—	—	
IV.	black	20±	21	—	+++	—	—	
IV.	black	20±	21	—	++	—	—	
					p-cresol catechol aniline†	p-cresol catechol	p-cresol aniline	p-cresol
V.	black	20±	24		++‡	+	+	—
VI.	white	20±	24		—	—	—	—
VII.	black	20±	20		++	+	—	—
VIII.	white	20±	20		—	—	—	—

* +++ indicates deep gray color through top half of solution when tyrosine is substrate, throughout when dopa is used.

† 1 cc. 0.1% p-cresol; 0.1 cc. 0.1% catechol; 0.5 cc. 1% aniline.

‡ ++ indicates medium brown color; +, reddish tan.

hours was considerably less than that reported by ONSLOW who found a charcoal black ring at the top of his test solutions, but was comparable to that secured by PUGH.

A third extract (IV) of black skin did not give any evidence of tyrosinase activity, either full strength or diluted. This same inactivity was found in extract III diluted 1:5 with water, although the undiluted extract was active. The fact that in this case at least dilution led to loss of tyrosinase activity suggests that in IV, where the method was apparently comparable some unknown difference in procedure led to lower concentration of enzyme in the extract as PUGH suggested in reference to the irregularity of her results. It is of interest in this connection that PUGH (1933) reported the same unexplained effect of dilution on the tyrosinase activity of mealworm extracts.

The difficulty in using dopa, under simple conditions, as a substrate for testing the presence of melanogenic enzymes in skin extracts is indicated in table 5 by its reaction in all cases, even without addition of other substances.

In an attempt to minimize the number of reactions involved in the tyrosinase tests, p-cresol was tried as a substrate known to be oxidized by tyrosinase without undergoing the complex series of secondary reactions which occur when tyrosine is used. As a possible means to increase the sensitivity of these further tests catechol and/or aniline were also added, in view of RICHTER'S report (1934) that (1) the direct action of tyrosinase is upon o-dihydric and not monohydric phenols, forming o-quinones, and that monohydric phenols serve as substrates for tyrosinase only after reacting with o-quinone to form dihydric phenols: and (2) that higher concentrations of o-quinone inhibit the enzyme. Hence catechol was added as a priming agent (that is, to supply o-quinone) in concentration so low that its own oxidation would not add enough color to the solution to suggest tyrosinase activity. This concentration should still be high enough to furnish adequate substrate for the initial action of the enzyme, if present. The aniline was added to combine with any possible, though unlikely, excess of o-quinone which might be formed.

Two black skin extracts tested under these conditions both showed the presence of tyrosinase (V and VII), with and without aniline. When catechol was omitted, only one of the extracts showed activity presumably because of the lack of enough priming material like catechol in the skin extract.

Two extracts from the skin of extreme spotted animals (VI and VIII), each tested simultaneously with one of the black extracts, did not show evidence of tyrosinase activity under any test conditions.

Since only one out of the total of five black extracts tested failed to react

positively for tyrosinase, the chance that two samples from other skin of similar enzyme content should both fail for the same unknown reason as the black is only about one in twenty-five (that is, the product of the chances that one should fail). Hence it may be concluded that probably, as ONSLOW reported originally, the white-furred areas of piebald mouse skin either lack, or have a much lower concentration of, the melanogenic enzyme present in the skin of colored animals. The data definitely confirm PUGH's finding that the enzyme is a true tyrosinase in that is operates without added peroxide and that, contrary to BLOCH's conclusion, mammals (or at least mice) thus do not lack the potentially melanogenic enzyme present in invertebrates. That this tyrosinase lies at least partly in the melanoblasts of the hair follicles and is actually the melanogenic enzyme of those cells is not proved by the present results, although it seems likely from the probable deficiency of the enzyme in the regions of skin where the follicles are not producing melanin. It is also yet to be proved that the colored-furred areas of spotted mouse skin have the same concentration of tyrosinase as the skin of unspotted mice, although this seems likely from the identity of the pigmentation. And it remains uncertain whether the white-furred areas of spotted mice are completely lacking in tyrosinase.

SUMMARY

The patterns of white-spotted mice have long been known to be rather diverse, even within presumably almost isogenic strains. The variability is limited, however; some regions of the skin bear pigmented fur in every individual; other regions always bear white fur, and it is only the intervening areas that have a variable behavior which may be characterized by the proportion of cases in which the hairs are pigmented.

1. Counts have been made of the proportion of animals bearing pigmented fur on each of 508 very small skin areas, in the presence of each of six combinations of "spotting" genes.

2. The proportions so obtained ("pigmentation frequencies") vary systematically over the skin surface, in the presence of any one genotype, forming a gradient field which drops off from the ear and rump regions towards the feet, tail tip, and mid-lumbar region.

3. The frequency fields of *ssKKEEFF* (4% of white fur), *ssKKEEff* (35%), *ssKkEeFf* (hybrid between 4% and 100% white strains) and *ssKkEeff* (35%×100%) are found to have the same general form but consistently different heights, the frequencies diminishing in the order indicated.

4. Similarly the frequency field of *SSkkeeff* (20%) is consistently higher than that of *Sskkeeff* (20%×100%); but neither of these two falls into the

previous series; that is, both have a somewhat different form than the fields obtained in the presence of *ss*, lower in the anterior dorsum and head, higher on the posterior dorsum and legs.

5. The difference in shape of pigmentation frequency field between genotypes containing *KEF* without *S* and those containing *S* without *KEF* is taken to indicate that *KEF* and *S*, though both acting ultimately on pigmentation frequency, have different sorts of primary effect.

6. The increase in pigmentation frequency of a given point from heterozygote to homozygote (*Sskkeeff* to *SSkkeeff; ssKkEeff* to *ssKKEEff*) is found to vary complexly with the value in the heterozygote.

7. But if the pigmentation frequencies are subjected to an inverse probability transformation (WRIGHT 1920) the values in heterozygote and homozygote are found to have an approximately linear relation. The transformation does not bring about an approach to constant difference between *Sskkeeff* and *SSkkeeff*, although it does for *ssKkEeff* and *ssKKEEff*, which is taken to be a second indication of a difference in nature of primary effects between the (*KEF*) and *S* genes.

8. Following WRIGHT (1920) the frequency fields are considered in terms of some hypothetical material whose concentration P at a particular point of the skin must equal or exceed a minimal value P_0 for the ultimate formation of pigment. Most aspects of the observed regional and genotypic variation of pigmentation frequency can be accounted for if it is assumed that:

(a) the P of a particular skin point varies somewhat from animal to animal of the same genotype;

(b) the average value either of P, or of P_0 or of both, in the presence of a given gene combination, varies systematically from point to point over the skin surface;

(c) *K*, *E*, *F* have a constant additive effect on either P or P_0 throughout the anterior and posterior halves of the body, the anterior increment being nearly three times as large as the posterior;

(d) *S* has some sort of multiplicative effect on one of the two variables, P or P_0.

9. As reported by ONSLOW (1915) but somewhat doubted from the results of subsequent workers, tyrosinase can generally be detected in extracts of skin which is forming pigmented hairs; but it is either absent, or greatly diminished, in extracts of those skin regions of spotted mice which are forming unpigmented hairs.

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