PREVENTION OF CONGENITAL OTOLITH DEFECT IN PALLID MUTANT MICE BY MANGANESE SUPPLEMENTATION[1]

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THERE are numerous genetic loci in mice which affect primarily pigmentation; SEARLE (1968) lists 44 loci with nearly 100 mutations. Eighteen (40%) of these loci possess mutant alleles with known pleiotropic effects. In some cases the pleiotropic effect has been traced to a biochemical, morphological, or developmental phenomenon, but no direct relationship has been demonstrated between the mutation's effect on pigmentation and the associated defect. The investigations presented in this paper suggest that some of these pleiotropic effects may involve subtle relationships between pigmentation and trace-element requirements.

The gene used in these studies is pallid (symbolized by *pa*) which was discovered in a wild population of mice (ROBERTS 1931). It was first identified as pink-eyed-2 because it was similar to but phenotypically and genetically distinct from pink-eyed dilution. In addition to the effect of *pa* on pigmentation, CASTLE (1941) observed that the viability of *pa* mice was considerably reduced under crowded conditions, and that they "tended to be nervous and jumpy." LYON (1951) showed that specific behavioral anomalies of these mice were associated with otolith defects within the inner ear.

As a result of these studies and of LYON's (1953, 1954, 1955a, 1955b) more detailed examination of *pa*, it can be described as a color mutation with pleiotropic effects on behavior, growth, and viability. The effect on pigmentation (including the absence of pigment from the membranous labyrinth) is fully penetrant in the homozygote recessive (*pa/pa*) whereas the effect on otolith formation is highly variable. Although the mass of otolith crystals (otoconia) may be reduced in or absent from any of the four otoliths, there is considerable asymmetry between left and right ears and between the utricle and saccule of the same ear. LYON (1954) elegantly demonstrated a significant effect of litter size on the penetrance of the otolith defect. She therefore postulated that the otolith defect "may be due to competition [*in utero*] for food substances, either general or particular, or for oxygen, space, etc."

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LYON's studies did not explain how *pa* caused these varied pleiotropic effects on pigment, otoliths, and growth, how the environment modified otolith development but not pigmentation, or how development resulted in considerable asymmetry. A clue was provided by studies involving manganese deficiency and development of the inner ear in rats (HURLEY *et al.* 1960). The congenital ataxia resulting from manganese deficiency (HURLEY, EVERSON and GEIGER 1958; HURLEY and EVERSON 1963) suggested that this trace element might be related to some of the morphogenetic mutations affecting the inner ears of mice (SIDMAN, GREEN and APPEL 1965; DEOL 1968). In a preliminary report, we showed that manganese deficiency in pregnant mice induced a defect of the inner ear which was morphologically and behaviorally indistinguishable from that of *pa* (ERWAY, HURLEY and FRASER 1966). However, manganese deficiency did not produce a phenocopy of the mutant gene's effect on pigmentation. As a complement to these findings, the ear defect, but not the pigment defect, of *pa* mice could be prevented by manganese supplementation during embryonic development. In the present paper, more experiments on the relationship between *pa* and manganese are reported. In addition, other pigment mutants were used to investigate the relationship between pigmentation and otolith development.

MATERIALS AND METHODS

Experimental animals: Mice were received in the spring of 1965 from The Jackson Laboratory where *pa* (V) was maintained in the C57BL/6J-*pa* inbred strain (N20 = 20th generation of backcrossing by Dr. E. S. RUSSELL). Experimental stocks were derived from these animals without attempting to continue brother-sister matings. Although some *pa* mice were also obtained from Professor MORRIS FOSTER at The University of Michigan, these stocks were originally derived from the same Jackson stock. No attempt has been made to maintain separately the Jackson and Michigan stocks.

Other mutant strains of mice have been studied for their effects on the inner ear or on pigmentation, or for their interactions with *pa*. Albino mice (BALB/cCrgl-*c*, F80) were crossed to *pa* in an effort to obtain F_2 progeny which were homozygous for both alleles (*c/c*; *pa/pa*). The mutation, viable dominant spotting (W^v), was received from The Jackson Laboratory in the C57BL/6J-W^v (~N75, RUSSELL) inbred strain. Since W^v/W^v individuals are sterile, these stocks were maintained by mating heterozygotes ($W^v/+$), to *pa/pa*. A $W^v/+$; *pa/pa* stock was established to test whether the effects of W^v would modify the incidence of the *pa* effect on otoliths.

Two other mutant strains of mice were also compared to *pa* for otolith development. Tilted head (*th*, XIII) was described by KELLY (1958) as a recessive gene with a high frequency of head tilting. This gene did not involve pigmentation, but it has been studied for its linkage to leaden (*ln*), a pigment-diluting gene, and to Splotch (*Sp*), a dominant spotting gene which is a homozygous lethal. The tilted-head stock (*th ln Sp/th ln* +) was obtained from Dr. LARSEN at The Oak Ridge National Laboratory. A mutant similar to *th*, unbalanced (CBA/H-*ub*), was reported by LYON and MEREDITH (1965) to involve an otolith defect similar to *pa* and to another gene, muted (*mu*, XIV, LYON and MEREDITH 1969), except that pigmentation was not affected. *Ub* (XIII) was a recessive mutation, always penetrant with otoliths lacking in both ears. This stock was obtained from Dr. M. F. LYON at The M.R.C. Radiobiological Research Unit, Harwell, England. The *th* and *ub* stocks were crossed and tested for allelism. Unfortunately, the *ub* stock has not been preserved by either Dr. LYON or by us, but the *th* stock is maintained at Oak Ridge.

Behavioral and morphological tests: The swimming criterion used for scoring manganese-deficient mice provided a most satisfactory approximation of the incidence of animals with morphological defects (ERWAY, HURLEY and FRASER 1970). Although animals affected according

to this test always exhibited some degree of otolith defect, a small percentage of mice were able to swim well despite a rather marked reduction in otoliths.

The otic capsules were dissected from the skull of mice of various ages, including fetuses and mature animals. The capsules were fixed in 10% formalin or in 70% ethyl alcohol, and dehydrated through grades of ethyl alcohol. They are cleared in methyl salicylate (oil of wintergreen) and observed as whole mounts. The four individual otoliths in each animal were scored (using polarizing lenses) on the basis of the apparent density of otoconia; they were categorically scored as 0 for no crystals or 3 for normal otoliths, with 1 and 2 being intermediate. The scores for every animal in each litter were pooled, the pooled value then being calculated as a percent of normal development. These values were averaged for all litters within each treatment to give a mean otolith score (M.O.S.).

Dietary levels of manganese: The *pa* mice were tested for the incidence of behavioral and otolith defects at several levels of dietary manganese. 1) Many of the mice were fed Purina Lab Chow which contained approximately 50 ppm of manganese. 2) Some mice were fed a purified diet (ERWAY, HURLEY and FRASER 1970) containing 45 ppm of manganese; such a diet was fed throughout the gestation period or for several successive pregnancies. 3) Some mice were fed the same purified diet except that it was supplemented with higher levels of manganese. 4) Other mice were fed the purified control diet (45 ppm), scored for vaginal plugs (day of plug = day 0 of gestation), and then fed the supplemented diet (1000 ppm), beginning on specific days of gestation to the end of pregnancy.

The absence of normal otoliths in *ub* prompted us to feed the purified diet to the CBA/H-*ub* mice, but this was discontinued when they became sick and died apparently as a result of eating the unusual diet. Therefore, in order to test *ub* mice for response to supplementation, manganese ($MnCl_2$) was added to the drinking water in a 0.1% solution.

RESULTS

Behavior of pallid mice: Most *pa* mice behaved normally under cage conditions, except that approximately 25% of them exhibited various degrees of head tilting. The seriously affected *pa* mice, when first dropped into water for the swimming test, frequently underwent a momentary tonic seizure. Then they became coordinated and swam in almost any direction, often undergoing tortuous spiralling or back-circling because of the retracted position of the head. After being removed to a solid surface, they often exhibited, temporarily, an exaggerated form of ataxia, head tilting, and sometimes rolled over repeatedly. Specific patterns of behavior seemed to be characteristic of individual animals.

By these criteria, 58 of the 99 *pa* mice tested were unable to swim. The correlation between inability to swim and otolith defects is demonstrated by the data in Table 1. Of the 26 animals with normal otoliths, all were able to swim normally, and 34 *pa* animals with no otoliths at all were unable to swim normally. Of 35 animals with a left-right asymmetry (no otoliths in one ear, normal in other ear), 28 swam normally, but seven were affected. Four animals appeared to swim normally with only one saccular otolith.

Individual *pa* mice exhibited degrees of left-right asymmetry in terms of otolith development, with no predominant direction to this asymmetry. There were far more cases of asymmetry within the same ear than Table 1 would indicate since the otolith scores of 1–3 were simplified to + for this correlation. In all cases such asymmetry was directional in that the utricular otolith was always as severely affected as, or more severely affected than, the saccular otolith. Individual *pa*

mice frequently exhibited an asymmetrical behavior pattern in which the head was constantly tilted to one side or the other.

LYON (1951) presented data for *pa* mice showing that the side tilted uppermost in young animals was the side more severely affected morphologically; however, she did not always find such a correlation in older mice. All of the manganese-deficient mice and many of the mutants were observed at 3–4 weeks of age. Thirty-four animals with four normal, or nearly normal, otoliths showed no head tilt (Table 2), whereas 27 of 61 animals with absolutely no otoliths did show some degree of head tilting. In contrast, 25 of 32 animals with a left-right morphological asymmetry (0/0 +/+) exhibited no head tilting. In two *pa* mice, there was an apparently negative correlation, i.e., the more severely affected side was tilted downward. The *ub* mice, which always had only a few otolith crystals, also failed to show any clearcut correlation between the direction of head tilting and morphological asymmetry.

Remedial effect of manganese supplementation: Our first report (ERWAY, HURLEY and FRASER 1966) indicated approximately 70% of *pa* mice were unable to swim normally when the breeding stocks had been maintained on laboratory stock diets (containing approximately 45–50 ppm of manganese). Maintenance of breeding stocks of *pa* mice on a purified diet containing 1000 ppm of manganese reduced the incidence of the swimming defect to zero. It was possible to obtain a more accurate estimate of the effect of manganese in the diet by maintaining *pa* mice on different levels of manganese throughout gestation. These results (Tables 3 and 5) are evaluated in terms of the mean otolith scores. These values increased roughly in proportion to the manganese level. In fact, the data in Table 5 show that the response was approximately linear ($r = 0.95$) over the range 45–2,000 ppm. This linearity is constrained by limits at approximately 0 ppm and 2,000 ppm. These data indicated a partially normal otolith development on the diet containing 1000 ppm of manganese, whereas the values for supplementation at 1500 ppm and at 2000 ppm showed an almost complete remedial effect even on a morphological basis.

TABLE 1

Correlation of swimming behavior and otolith defects in pallid mice

State of otoliths		Swimming behavior	
u/s	u/s‡	Normal	Affected
		Number	Number
+/+	+/+	26	0
0/0	+/+	28	7
0/0	0/+	4	0
0/0	0/0	0	34
		—	—
		58	41

‡ Coded score: u = utricular otolith in left or right ear
s = saccular otolith in left or right ear
+ = presence of any otoconia
0 = no trace of otoconia

TABLE 2

Asymmetrical posture morphological defects in mice

Otoliths[1]		Manganese-deficient head tilt		Pallid[2] head tilt		Unbalanced[3] head tilt	
		With	Without	With	Without	With	Without
		Number	Number	Number	Number	Number	Number
+/+	+/+	0	20	0	14	0	0
0/0	0/0	12	9	10	22	5	3
0/0	+/+[4]	1 pos.	8	4 pos. & 2 neg.	17	0	0
0/—	0/—	0	2	0	3	7	45
0/0	0/—	0	0	1 pos.	0	6 pos. & 6 neg.	4
		—	—	—	—	—	—
		13	39	17	56	24	52

[1] 0 = no trace of otoconia; — = only a few relatively large crystals as normally observed in unbalanced mice; + = any otolith having numerous otoconia.

[2] LYON (1951) reported evidence for a positive correlation between the side tilted uppermost and the side more severely affected morphologically. Both positive (pos.) and negative (neg.) evidence of this sort was observed in the present data.

[3] Unbalanced mutants were never observed to have normal otoliths, only rarely having more than 20 large crystals.

[4] All asymmetrical pairs of ears, including both left and right affected ears, were combined.

TABLE 3

Manganese supplementation of pallid mice throughout gestation

Synthetic diet Mn (ppm)	Litters	Progeny	Mean litter size	M.O.S.[1] ± S.E.		
	Number	Number		Percentage		
45	23	123	5.3	10.3 ± 2.8	*	
100	9	56	6.2	21.7 ± 2.0	*	
500	23	102	4.4	61.6 ± 4.0[2a]	63.1 ± 3.4	*
1,000	7	36	5.1	68.1 ± 5.8[2b]	*	
1,500	7	38	5.4	94.7 ± 1.9[2c]	96.1 ± 1.1	
2,000	10	50	5.0	97.0 ± 1.3[2c]		

* Differences between adjacent values significant at $P \leq 0.001$; data pooled when differences were not significant ($P \geq 0.05$).

[1] M.O.S.—Mean otolith score calculated from score for each litter:

$$\left(\frac{\text{Sum of otolith scores observed per litter}}{\text{Total otolith score expected per normal litter}}\right) \times 100$$

[2] The severity of the otolith defect may be judged according to the designations used in Tables 1 and 2, all animals with 0/0 0/0 being unable to swim and some of those with 0/0 +/+ exhibiting swimming defects.

a. 9 individuals 0/0 0/0; 17 0/0 +/+

b. 1 individual 0/0 0/0; 4 0/0 +/+

c. No single otolith was scored as 0.

Having established a dose-response effect when the same diet was fed throughout gestation, it was desirable to obtain some indication of the effective period for manganese supplementation. The values (Tables 4 and 5) indicate that supplementation beginning on or after day 14 of gestation was ineffective in increasing the mean otolith score as compared to maintenance on 45 ppm, yielding a mean of 11.5% of normal otolith development. Initiation of the supplement on days 12 and 13 yielded a significant increase, but these values were only intermediate (31%) to the unsupplemented and completely supplemented values (~ 72%). Initiation of the supplement as early as day 10 and 11 produced results comparable to supplementation (1000 ppm) throughout gestation. These results also showed an approximately linear ($r = 0.93$) response when supplementation on day 16 = 1, day 15 = 2, day 14 = 3, etc. However, such linearity may have little biological significance except over a narrow time range, presumably from day 11 through day 13 or 14.

Allelism of tilted head and unbalanced: The *th* and *ub* mutant mice were tested for swimming ability and examined for otoliths. All of them exhibited considerable swimming difficulties except for the unusual ability of some of them to remain nose-up in the water. Indeed, as LYON and MEREDITH (1965) had reported for *ub*, all of these mice lacked normal otoliths in both ears. However, a very limited number of crystals were present, usually in one or both of the saccules, but seldom in the utricles. These crystals were many times larger (upwards of

TABLE 4

Timed manganese supplementation (1,000 ppm) of pallid mice during gestation

Fetal age at initiation of supplement†	Litters	Progeny	M.O.S.‡ ± S.E.	
Days	Number	Number	Percentage	
Unsupplemented	23	123	10.3 ± 2.8	11.5 ± 2.1*
16	8	38	16.1 ± 7.6	
15	5	29	13.4 ± 1.7	
14	7	39	9.1 ± 2.7	
13	6	24	27.0 ± 7.5	31.0 ± 5.5*
12	3	15	39.0 ± 6.2	
11	3	14	69.6 ± 10.7	71.2 ± 4.6*
10	2	10	84.5 ± 12.5	
Throughout gestation	7	36	68.1 ± 5.8	

* Each M.O.S. was tested for significant difference from "unsupplemented" or "throughout gestation" values. When the differences were not significant ($P \geq 0.5$) the data were pooled to give a grand M.O.S. value. The differences between the three values were highly significant ($P \leq 0.001$).

† Pregnant females were transferred from the synthetic diet containing 45 ppm Mn to one containing 1,000 ppm, beginning on the indicated day after observation of vaginal plugs, and were continued thereafter on the supplementation diet.

‡ M.O.S.—Mean otolith score calculated as:

$$\frac{\text{Sum of otolith scores observed per litter}}{\text{Total otolith score expected per normal litter}} \times 100$$

TABLE 5

Distribution and analysis of otolith scores for supplemented pallid mice

Experiment*	Number with otolith scores				Number		Linear regression statistics
	0	1	2	3	Total	Mean	
ppm Mn							
45	415	21	47	9	492	0.29	
100†	138	28	47	5	218	0.63	r = 0.95
500	73	46	124	133	376	1.84	a = −332 ppm
1,000	19	24	29	66	138	2.20	b = 656 ppm
1,500	0	5	12	135	152	2.82	$s_{y.x}$ = 220 ppm
2,000	0	6	4	192	202	2.92	
day							
16‡	133	4	14	7	158	0.34	
15	94	3	14	5	116	0.40	r = 0.93
14	135	5	9	5	154	0.25	a = 1.58 days§
13	59	11	13	13	96	0.79	b = 2.24 days
12	27	5	17	11	60	1.20	$s_{y.x}$ = 0.74 days
11	8	9	11	28	56	2.05	
10	3	2	6	29	40	2.53	

* Data from same litters as shown in Tables 3 and 4; all otolith scores pooled within litters and within treatments.

† Distributions were compared by means of a 2 × R contingency chi-square; all adjacent values were significantly different ($P \leq 0.01$); including data for 500 and 1,000 ppm and for 1,500 and 2,000 ppm.

‡ On the basis of a 2 × R contingency chi-square analysis, the following conclusions were made:
a. Days 14, 15, and 16 were not significantly different from 45 ppm.
b. Days 10 and 11 were not significantly different from 1,000 ppm.
c. Days 12 and 13 were not significantly different from each other, but they were both significantly different ($P \leq 0.001$) from 45 ppm and from 1,000 ppm.

§ Linear regression analysis in which supplementation on day 16 = 1, day 15 = 2, etc.

0.2 mm in length) than the ordinary crystals (approximately 10–20 microns) present in the normal otolith matrix.

Since the phenotypic expressions of *th* and *ub* were indistinguishable, they were tested for allelism. The F_1 progeny of reciprocal crosses failed to exhibit complementation among 50 offspring from 8 litters. The F_1 offspring exhibited both the swimming defect and the abnormal crystals in place of the normal otoliths. Therefore, *th* and *ub* may be considered as alleles of each other, and *ub* should be symbolized as th^{ub}.

Some pregnant CBA/H-*ub* mice were given tap water, and others were given the manganese-supplemented water (0.1% $MnCl_2$). The offspring were scored for swimming and for number of abnormal crystals. The control animals exhibited an average of 3.8 of the abnormal crystals per animal (N = 16). The supplemented animals possessed an average of 7.2 crystals per animal (N = 20). This was a significant increase in crystals, and there was a concomitant improvement in the swimming ability of the supplemented animals. Nevertheless, normal dense otoliths were never observed, and the crystals were clearly abnormal.

Test for genetic interactions: Two experiments were performed to clarify the

possible relationship of the effect of *pa* on pigment and on otolith development. Certain other mutant genes were used in an attempt to interfere with different aspects of melanogenesis, i.e., *c* presumably disrupts tyrosinase activity and W^v presumably inhibits melanoblast migration into the white areas, particularly of the skin (WOLFE and COLEMAN 1966). One might thus predict that *c* would suppress the effect of *pa* on otolith development if *pa* exerted its effect on manganese availability via an alteration of the melanin structure (e.g., greater chelating capacity for manganese). In a similar manner, W^v might be expected to suppress the effect of *pa* on otolith development if the removal of melanocytes from other regions of the body did not withdraw manganese from circulation.

The crosses between *c* and *pa* stocks produced some F_2 progeny which were albino, exhibited the swimming defect, and lacked otoliths. Since no other albino stock has been observed to exhibit the behavioral defect, these F_2 animals were presumed to be homozygous for the *pa* gene (*c/c*; *pa/pa*). A few dozen progeny from these selected F_2 offspring were observed with a high incidence of the inability to swim. No data were obtained to compare either the swimming defect or the otolith defect in *c/c*; *pa/pa*; vs. c^+/c^+; *pa/pa*. Nevertheless *c* did not suppress the effect of *pa* on otoliths to any appreciable degree.

pa and W^v were mutually epistatic in their respective effects on pigmentation, i.e., *pa* suppressed pigmentation of the eyes and inner ears, and W^v/W^v suppressed all coat color, hence the double homozygote (*pa/pa*; W^v/W^v) was a mock albino. Otolith scores are presented for the three genotypes in Table 6. There is no evidence of any correlation between the degree of pigmentation of the coat and otolith development.

DISCUSSION

The results show that normal dietary levels of manganese are insufficient for otolith development in *pa* mice. In non-pallid mice, otolith defects were produced when the dietary manganese level was of the order of 3 ppm (ERWAY, HURLEY

TABLE 6

*Interaction of pallid and viable dominant spotting**

	Genotype		
	+/+;*pa/pa*	$W^v/+$;*pa/pa*	W^v/W^v;*pa/pa*
Phenotype	†	‡	§
Number observed	24	58	24
Otolith score (percentage)	43 (12)¶	30 (16)	45 (14)
Standard deviation‖ (Percentage)	29	18	32

* $W^v/+$;*pa/pa inter se* matings produced 17 litters, 106 progeny (mean litter size = 6.2).
† This genotype is a typical pallid; pale coat, pink eyes, no spots.
‡ This genotype has pink eyes, extensive spotting on the head and belly, and a very much diluted general coat color.
§ This is a "mock albino," having no eye, ear, or coat color.
¶ The values in parentheses indicate the number of litters having at least one individual of this genotype from which the mean otolith score was calculated.
‖ The greatest difference in means was not significant ($t = 1.56$; $df = 28$; $P > 0.05$).

and FRASER 1970). By contrast, in *pa* mice, similar anomalies were seen with 45 ppm, and the mice required 1500 to 2000 ppm of dietary manganese for normal development. It appears that *pa* mice may have a dietary manganese requirement which is a few hundred times higher than normal.—Therefore, we conclude that manganese is the "particular food substance," as postulated by LYON (1954), for which *pa* embryos compete.

There is considerable evidence (COTZIAS 1962) that levels of manganese in the body are efficiently regulated by the liver. One might assume, therefore, that *pa* mice possess some abnormality in ability to absorb, transport, or otherwise regulate manganese levels in the body. However, there is evidence that *pa* does not affect the general availability of manganese. First of all, LYON (1953) found no evidence of a maternal effect, i.e., incidence of the otolith defect was the same among *pa* offspring of reciprocal backcrosses. The absence of a generalized effect is also supported by the observation that neither *c* nor W^v altered the effect of *pa* on otoliths, in spite of the absence of pigment or pigment cells from the rest of the body. Furthermore, autoradiography shows that *pa* affects incorporation of $^{35}SO_4$ into the otolith matrix but not into the surrounding otic cartilage, whereas manganese deficiency affected both tissues (ERWAY 1968). Thus, *pa* apparently causes a very highly localized state of manganese deficiency. This conclusion is also supported by biochemical and electron microscopic comparisons of liver mitochondria from manganese-deficient and *pa* mice (HURLEY, THERIAULT and DREOSTI 1970). Whereas manganese deficiency produced marked changes in mitochondrial structure and function, *pa* exhibited no such effects.

LYON (1955a, 1955b) reported that the otolith matrix and crystals were first visible in the mouse between days 15½ and 16½ of gestation, an observation which we have confirmed (ERWAY 1968). In the experiments presented here, manganese supplementation of *pa* mice was ineffective on the 14th day of gestation, or later, in preventing otolith defects. By comparison, manganese supplementation on the 14th day of gestation was partially effective in manganese-deficient mice, but it was not effective on the 15th day (ERWAY, HURLEY and FRASER 1970). This difference of one day may be related to an inability of *pa* to accumulate manganese in the inner ear possibly because of the absence of melanocytes. Both *pa* and manganese-deficient mice showed an intermediate response to manganese supplementation on the 12th and 13th days of gestation, and the maximal response when supplemented on or before the 11th day. Whatever the specific role of manganese in otolith development may be, it appears to be required several days prior to the appearance of the otolith matrix and otoconia.

These studies may be of more general significance than might be assumed from the specific effects of *pa*. Several other pigment mutations with associated otolith defects are known. The *mu* gene in mice produces a dull brown fur, lightly pigmented eyes, as well as otolith defects (LYON and MEREDITH 1969). Another mutant called mocha causes a slightly darker coat color than *pa*, no visible eye pigment, and a behavior like *pa* (LANE 1967). These three mutations are not allelic.

A search for analogous genetic defects in other species has provided several

interesting cases. A grey-loco (*g-lo*) condition was observed in chukar partridges (ABBOTT and ABPLANALP, personal communication). Newly hatched chicks with this condition possessed defective otoliths as well as reduced pigmentation within the labyrinth (ERWAY 1968). CRAIG (1969) obtained 14 *g-lo* segregants from chukar-partridge hens supplemented with two levels of manganese and 27 from a group fed the same diet, but without added manganese. While the addition of manganese to the maternal diet did not prevent otolith defects, an analysis of individual otolith scores indicated a significant difference between groups in the degree of abnormality. Supplementation with manganese appeared to reduce the number of completely missing otoliths, resulting in an increased number with partial development. This evidence supports the probable analogy between *pa* and *g-lo*.

A genetic mutation known as pastel in mink is also associated with behavioral defects described as screw neck (SHACKELFORD and COLE 1947). Evidence (ERWAY and MITCHELL 1970) now shows that pastel mink also lack inner ear pigment and have extensive otolith defects. Another mutant of probable analogy was described in rabbits by MAGNUSSEN (1960). Ocular albinism was a mutation whose pigment effects were apparently restricted to the eye (little or no effect on coat color). However, head tilting, typically associated with this mutant, was attributed to labyrinthine dysfunction. The ears of ocular albino rabbits also showed complete absence of pigment and defective otoliths (ERWAY 1968). It is postulated that a sex-linked form of ocular albinism in man (FALLS 1951) may also be analogous to the cases cited above.

Such a correlation between these pigment mutations and development of otoliths suggests an unusual basis for the pleiotropism of gene effects. Moreover, since the otolith defect can be prevented, whereas pigmentation remains unchanged, the relationship between pigmentation and manganese is especially intriguing. Although the absence of pigment in the labyrinth suggests a causal relationship to otolith development, the correlation is not perfect since some *pa* mice have normal otoliths.

As the result of these studies, we have developed the hypothesis that the presence of melanocytes in the inner ear serves as a reservoir for manganese. Therefore, the inner ear of *pa*/+ mice should contain more manganese than that of *pa*/*pa*, a possibility to be examined in detail. A similar hypothesis, suggesting that melanin may chelate manganese, was advanced to explain the correlation between manganese concentration and pigmented tissues (COTZIAS, PAPAVASILIOU and MILLER 1964; VAN WOERT, NICHOLSON and COTZIAS 1965). Our evidence indicates that albino animals, presumably containing amelanotic melanocytes in the inner ear, also possess the manganese reservoir. This suggests that some premelanotic stage of the melanocyte, perhaps its melanosomes, is critical to the availability of manganese for otolith development. One may therefore argue that *pa* is, in effect, a spotting mutant with regard to the membranous labyrinth. Indeed, electron microscopy of the *pa* inner ear failed to reveal any melanocytes, even amelanotic ones, whereas typical but fewer melanocytes were found in the retina (THERIAULT and HURLEY 1970).

Even though six different mutations in four species exhibit the apparent re-

lationship between pigment and otolith development, it is remotely possible that a gene for otolith development is closely linked to the respective pigment genes. In spite of repeated backcrossing of *pa*/+ to *pa*/*pa*, however, neither LYON (1953) nor ourselves has ever discovered any dissociation of the otolith defect from the pigment defect. The more simple hypothesis, therefore, seems to be that certain pigment mutations may produce subtle effects on trace-element metabolism, which in turn may alter the delicate balance of enzyme systems requiring these metallic cofactors. In the case of *pa*, the evidence indicates that the synthesis of mucopolysaccharides, comprising the matrix in which otoconia are formed, is affected (ERWAY, HURLEY and FRASER 1970).

This hypothesis of a relationship between melanocytes and trace elements also offers a possible explanation of some of the wide variety of pleiotropic effects associated with numerous pigment mutations (SEARLE 1968). Biologists have long been intrigued by the rather ubiquitous distribution of melanocytes throughout the vertebrate body, and by their accumulation in certain regions such as the labyrinth, Harderian gland, substantia nigra, etc., where pigment had no possible relationship to light or ultraviolet absorption.

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SUMMARY

A recessive pigment mutation, pallid (*pa*), has a pleiotropic effect on otolith development in mice. Manganese deficiency produces a phenocopy of the *pa* otolith defect, suggesting that manganese is involved in the effects of the *pa* gene. Manganese supplementation of pregnant *pa* mice prevents the otolith defect if fed in high concentrations and at critical times during embryonic development. Neither manganese deficiency nor supplementation alters pigmentation. Evidence is presented that melanocytes, normally present near the otoliths, may be a reservoir for manganese. This hypothesis is supported by six cases of analogous defects other than *pa*.—Two other mutations in mice are only partially analogous to *pa*. Tilted head and unbalanced are identified as alleles of each other; they do not affect pigmentation, and their otolith defects are distinct from *pa*.

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