Congenital Ataxia and Otolith Defects Due to Manganese Deficiency in Mice [1]

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ABSTRACT Manganese deficiency in mice caused congenital ataxia in some progeny. The most diagnostic means of detecting the ataxia was to place all mice in a tank of water and observe their ability to maintain a normal, upright position. The incidence of ataxia increased approximately in proportion to the duration and severity of the manganese-deficient state of the mother. Cleared otic capsules revealed that otoliths were reduced in size, or were absent, in many of the manganese-deficient progeny. Supplementation of the manganese-deficient females after day 14 of gestation was ineffective in reducing the incidence of the otolithic effect. Supplementation between days 12 and 14 was only partially effective, whereas supplementation starting on day 11 was sufficient for completely normal development of otoliths. The absence of otolithic crystals is attributed to a defect in the synthesis of the organic matrix which appears to be composed of acid mucopolysaccharides. The absence of otoliths was also found in manganese-deficient chicks and presumably explains the long-standing reports of ataxia due to manganese deficiency in poultry and other domestic animals.

Manganese is known to be an essential trace element for growth and reproduction in many animals, including mice, rats, chickens, pigs and guinea pigs (1–6). It is particularly important for the growth and development of the skeleton during both embryonic and juvenile periods of growth (7–13). The effects on skeletal growth appear to be mediated by changes in mucopolysaccharide synthesis, involving the cartilaginous matrices of forming bones (14–16).

In addition to the skeletal abnormalities, manganese deficiency leads to congenital ataxia in all of the above-named animals (17–20). Delayed righting reflexes and ataxic behavior in rats have been associated with anomalous ossification of the inner ears and with abnormalities of the membranous labyrinth (21–23). A preliminary report of the present studies indicated that the ataxia could be attributed to the absence of otoliths from the inner ears of manganese-deficient mice (24). This finding has since been confirmed in rats and guinea pigs (25, 26).

These nutritionally induced defects indicated a possible relationship to genetic defects associated with certain mutant genes in mice. More than thirty mutations are known to affect the morphogenesis or physiology of the inner ear (27). Four of these mutations have been reported to interfere with otolith development with little, if any, other effect on the inner ear. A mutant known as *pallid* because of its effect on pigmentation has been well described for its ataxic behavior and absence of otoliths (28, 29). The behavior and otolithic defects associated with the *pallid* mutant mice were indistinguishable from those of manganese-deficient mice, thus indicating a possible relationship between this gene and manganese metabolism. *Pallid* mice were supplemented, therefore, with high levels of manganese during pregnancy with the result that the congenital ataxia, normally associated with the gene, was prevented (24). The mutant gene itself was unaltered by the treatment, and the effects of the gene on pigmentation were not changed.

This paper presents a detailed report on the studies of manganese deficiency in mice

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and its congenital ataxia resulting from abnormal otoliths. The effects of supplementation of manganese-deficient mice, beginning at various times during gestation, are described. The observation of otolithic defects in manganese-deficient chicks is also included.

MATERIALS AND METHODS

Experimental animals. The majority of the manganese deficiency studies have used crossbred mice which were originally derived from a four-way cross of the inbred strains, AKR/J, C57BL/6J, C3H/J and DBA/2J.[4] The mice used in experiments A and B were unselected stocks from the F_2 and subsequent generations of these crossbred mice. In addition, data are reported for experiment C using C57BL/10J mice.

From previous studies of manganese deficiency in White Leghorn laying hens [5] approximately two dozen normal and manganese-deficient chicks were available for study. The manganese-deficient chicks were ataxic or were dead in the shell at the time they were prepared. The specimens were cleared and stained with alizarin red, and the inner ears were examined for otoliths.

Behavioral criteria. Previous work on body-righting reflexes in manganese-deficient ataxic rats showed that they eventually learned to right themselves in air, but were unable to do so in water (25). In the present studies, therefore, the ability of mice to maintain a normal upright position in water was used as a criterion of ataxia, and is referred to simply as swimming ability.

Dissection and morphological criteria. The otic capsules were dissected from decapitated newborn, weanling and adult mice. The skin of the head was pulled anteriorly so as to tear the ear canals away from the external meatus. Surrounding muscles were removed to reveal the tympanic bulla. The otic capsule was separated from the surrounding bones and removed intact. The tympanic bulla and ossicles were often removed from the freshly dissected capsules without damage to the labyrinth. More precise details for dissection are given elsewhere.[6]

The otic capsules were fixed in 70% ethyl alcohol, dehydrated in 95% and 100% ethyl alcohol, and cleared in methyl salicylate (oil of wintergreen). The cleared otic capsules, submerged in the oil, were studied under a dissecting microscope equipped for bright transillumination. The use of attached polarizing lenses was also of great advantage in demonstrating the crystalline nature of the otolithic structures. The thin, bony labyrinth of the mouse ear became transparent in oil of wintergreen, revealing clearly the membranous labyrinth, its associated canals, pigmentation, ossicles and otoliths.

The otoliths were scored on a four-grade scale: 3 = an otolith indistinguishable from normal; 2 = otolith noticeably reduced in size; 1 = an otolith with only traces of crystalline material; and 0 = no trace of crystals present. Such a score was applied to both utricular and saccular otoliths in the left as well as the right ear of each mouse. An animal with both otoliths in each ear completely normal would thus have a score of 12 or ($3 \times 2 \times 2$). On the basis of these otolith scores of individual animals, a percentage of normal development was calculated for each litter:

$$\text{Percentage of Normal Development} = \frac{\text{sum of scores for the litter}}{\text{no. of individuals in litter} \times 12} \times 100$$

All percentages for each treatment were then averaged to give a mean otolith score (M.O.S.) in percentage of normal development.

Histological examination of the inner ear was made by staining with toluidine blue.

Diets and feeding regimes. A purified diet was prepared from casein, dextrose and oil and was fortified with vitamins and appropriate salt mixtures (see table 1 for formula). Periodic spectrophotometric assays confirmed that the diets contained the levels of manganese specified.

Female mice (experiments A, B, and C) were fed purified diets (deficient diets =

[4] Professor G. E. Bradford, Department of Animal Science, University of California, Davis, provided mice from the control stocks used for his studies of selection for litter size and body weight (30).

[5] Professor C. R. Grau, Department of Poultry Husbandry, University of California, Davis, collaborated in these studies. Laying hens were fed a manganese-deficient diet and their eggs were used for studies of ataxia in the chicks.

[6] Erway, L. C., Jr. 1968 Genetic and Nutritional Interactions of Some Pigment Mutants, Manganese Metabolism and Otolith Development. Ph.D. Dissertation, University of California, Davis. (Dissertation Abstracts 29: 11, 1969.)

TABLE 1

Formula for purified diet used in manganese deficiency studies in mice

Final diet mix		Vitamin mix		Salt mix	
	%		g/kg		g/kg
Dextrose	54.5	Folic acid	0.030	$CaCO_3$	300.0
Casein [1]	30.0	Biotin	0.125	K_2HPO_4	321.0
Corn oil	8.0	Vitamins A and D_3 [2]	0.230	NaCl	168.0
Salt mix	6.0	(each 325,000 IU/g)		$MgSO_4 \cdot 7H_2O$	125.0
Vitamin mix	1.5	*p*-Amino benzoic acid	0.500	$CaHPO_4$	60.0
	100.0	Riboflavin	0.500	$FeSO_4 \cdot 7H_2O$	25.0
		Menadione	1.250	KI	0.80
		Nicotinic acid	1.500	$ZnCO_3$	0.25
		Pyridoxine	1.500	$CuSO_4 \cdot 5H_2O$	0.30
		Thiamin·HCl	1.500	$MnSO_4 \cdot H_2O$	varied [4]
		Vitamin B_{12} [3] (1 mg/g)	1.500		1,000.30
		Vitamin A [2] (325,000 IU/g)	2.100		
		Ca pantothenic acid	2.500		
		Ascorbic acid	5.000		
		Vitamin E [2] (125,00 IU/g)	21.400		
		Inositol	25.000		
		Choline chloride	50.000		
		Dextrose to make up	1,000.000		

[1] Casein (Purified High Nitrogen) was obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio.
[2] Vitamin mixtures were obtained from Hoffmann-La Roche, Inc., Nutley, N. J.
[3] Vitamin B_{12} in mannitol was obtained from Merck and Co., Inc., Rahway, N. J.
[4] Manganese sulfate was added in the amount of 51.5 mg per kilogram of salt mixture for preparation of the diet containing 1 ppm manganese. Multiples of this amount were used for higher levels of manganese.

1 ppm and 3 ppm Mn; control diet = 45 ppm Mn) (table 1) beginning at 6 to 8 weeks of age. They were fed the diet for 3 or 4 weeks before mating, and for the rest of the experiment. The offspring were scored for swimming behavior at 3 or 4 weeks of age. A representative group of offspring in experiments A and C were killed and their ears were prepared and scored for otoliths. All offspring in experiment B were scored for both swimming and otoliths.

In experiment D, the feeding regime was modified to study the effects of prolonged manganese deficiency. Some of the mice of the first generation (in experiment A, table 2) were fed the manganese-deficient diet (3 ppm) throughout their lifetime. These mice produced a second generation of manganese-deficient mice. In turn, some of the second generation mice were fed the deficient diet and allowed to reproduce. This procedure was repeated with mice continuously kept on the deficient diet until the sixth generation was produced and scored. These mice were maintained in six or eight cages with three or four females per cage, without particular reference to abnormalities, reproductive performance, or inbreeding. However, attempts were made to randomize the matings by interchanging males among the cages. Only the first few litters produced in each succeeding generation were used to maintain the stocks, all available females being used in the fifth generation.

Supplementation studies. Experiment E was designed to obtain information concerning the time at which manganese was required for normal otolith development. Normal female mice with young litters (1 to 2 weeks of age) were fed the manganese-deficient diet (1 ppm). When the litters were weaned, the young females in the litters were fed the same diet, and subsequently mated at 8 to 10 weeks of age. They produced two successive litters which were studied. The offspring were killed shortly after birth, dissected and prepared for scoring otoliths. The second set of matings was determined by vaginal plugs, and the pregnant animals were transferred from the deficient diet to a similar but manganese-high diet (1,000 ppm) beginning 10, 11, 12, 13, 14 or 15 days after mating. They were fed this diet for the remainder of the gestational period.

RESULTS

Litter size and survival. Five independent experiments were performed to determine the nature and extent of the congeni-

TABLE 2

Birth, survival and incidence of swimming defect in control and manganese-deficient mice

Progenies scored		Litters	Born	Survival to 21 days		Swimming affected	
	Order [1]	*No.*	*No.*	*No.*	*%*	*No.*	*%*
		Controls (45 ppm Mn)					
Expt. A	1st	7	47	35	76	0	0
	2nd	8	54	49	91	0	0
	3rd	5	41	39	95	0	0
		20	142	123			
Expt. B	1st	13	103	90	87	0	0
	2nd	13	116	103	89	0	0
	3rd	9	85	63	74	0	0
		35	304	256			
Expt. C	1st	8	62	46	74	0	0
	2nd	5	39	27	69	0	0
		13	101	73			
		Manganese-deficient (3 ppm)					
Expt. A	1st	8	71	33	46	3 [4]	9
	2nd	7	52	45	86	11 [4]	24
	3rd	6	49	38	77	8 [4]	24
		21	172	116 **		22 (8) [5]	
Expt. D, 2nd gen. [2]	1st	8	63	53	84	44	83
Expt. D, 6th gen. [3]	1st	21	175	155	88	9 [6]	6
Expt. B	1st	15	96	82	85	0 [4]	0
	2nd	14	128	118	92	15 [4]	13
	3rd	11	101	82	81	7 [4]	8
		40	325	282		22 (10) [5]	
Expt. C	1st	8	60	54	90	2 [4]	4
	2nd	5	41	27	66	17 [4]	63
		13	101	81		19 (10) [5]	
		Manganese-deficient (1 ppm)					
Expt. A	1st	7	42	27	64	1 [4]	4
	2nd	8	48	31	65	19 [4]	61
	3rd	6	40	28	70	28 [4]	100
		21	130	86 **		48 (7) [5] ‡	
Expt. B	1st	15	114	78	68	16 [4]	20
	2nd	14	130	117	90	41 [4]	35
	3rd	11	95	70	74	25 [4]	36
		40	339	265 *		82 (9) [5] ‡	
Expt. C	1st	8	55	24	44	8 [4]	33
	2nd	6	42	24	57	14 [4]	58
		14	97	48 **		22 (6) [5] †	

[1] Successive litters from female mice.

[2] Some members of 1st litters (expt. A) were reared on manganese-deficient diet (3 ppm) until they reproduced the second generation.

[3] Some members of succeeding generations were continuously fed the manganese-deficient diet (3 ppm). Litters were scored for both swimming and otoliths. Only 14 of 155 mice exhibited any morphological defect in otoliths.

[4] A 2×3 (2×2 for expt. C) contingency chi-square comparison of data pooled for 3 ppm and 1 ppm within each experiment, indicates that there was a significant difference ($P \leq 0.01$) between litters.

[5] The value in parentheses indicates minimum number of affected animals required to be significantly different ($P \leq 0.01$) from the respective control animals.

[6] A 2×2 contingency chi-square comparison indicates that the incidence of affected animals in the sixth generation is significantly lower ($P \leq 0.001$) than the incidence in the second generation.

* $P \leq 0.05$, ** $P \leq 0.001$; a 2×2 contingency chi-square comparison indicates that survival of manganese-deficient mice was different from survival of respective control animals.

† $P \leq 0.10$, ‡ $P \leq 0.001$; a 2×2 contingency chi-square comparison indicates that incidence of swimming defect at 1 ppm was lower than incidence of defect at 3 ppm for mice of same experiment.

TABLE 3

Morphological score of otoliths in control and manganese-deficient mice

Treatment	Litters scored	Animals scored	Affected [1]		M.O.S.[2]
Order	*No.*	*No.*	*No.*	*%*	*%*
Expt. B, control (45 ppm)					
	35	256	0	0	100
Expt. B, manganese-deficient (3 ppm)					
1st	15	82	2	2	99±0
2nd	14	118	24	20	85±5 †
3rd	11	82	15	18	92±5
	40	282	41 (10) [3]		93±3
Expt. B, manganese-deficient (1 ppm)					
1st	15	78	29	37	81±7
2nd	14	117	54	46	61±11
3rd	11	70	38	54	67±9
	40	265	121 * (9) [3]		71±6 ‡
Expt. E. manganese-deficient (1 ppm) [4]					
1st	57	300	237	79	52±4
2nd	9	49	49	100	15±11 †

[1] Any animal with reduction in size or number of otoliths.
[2] M.O.S. = Mean otolith score as a percent of normal development based on total score per litter (mean ± SEM).
[3] The value in parentheses indicates the minimum number of affected animals required to be significantly different ($P \leq 0.01$) from control animals.
[4] These data are obtained from experiment E in which the parents of these offspring were reared on manganese-deficient diet (1 ppm). Offspring were killed and scored shortly after birth instead of at 3 or 4 weeks of age.
* $P \leq 0.001$; a 2×2 contingency chi-square comparison indicates that incidence of otolith defect a 1 ppm is lower than incidence of defect at 3 ppm.
† A *t* test of mean otolith scores within treatments indicates significant differences between litters: Expt. B, $P \leq 0.02$; Expt. E, $P \leq 0.001$.
‡ $P \leq 0.002$; a *t* test of pooled scores indicates that the M.O.S. at 1 ppm is significantly lower than at 3 ppm.

tal ataxia induced in mice by manganese deficiency. The results of these experiments have been compiled in tables 2 and 3.

At levels of manganese deficiency attained in most of these experiments there was little or no effect on mean litter size. However, survival to weaning age was significantly lower for some of the manganese-deficient offspring, especially at 3 ppm and 1 ppm in the crossbred mice of experiment A, and 1 ppm for the inbred mice of experiment C. The survival of crossbred mice of experiment B was reduced at 1 ppm ($P \leq 0.05$), a fact which may be related to the generally lower incidence of the swimming defect in this experiment.

In addition to the effect on survival, prolonged exposure to manganese deficiency first reduced male fertility and some undetermined aspect of female reproduction. For example, the crossbred mice did not reproduce after one complete generation on the diet containing 1 ppm of manganese, whereas those fed a diet of 3 ppm manganese did reproduce.

Swimming ability. Primary emphasis was placed upon the behavioral defects, particularly the inability to swim (see fig. 1) and upon morphologically detectable defects involving the otoliths (see fig. 2). Control animals fed the same purified diet, except that it contained 45 ppm manganese, never exhibited behavioral abnormalities or otolithic defects. The incidence of the swimming defect tended to increase with successive litters and with the severity of the dietary deficiency (table 2). The data for the inbred C57BL/10J mice were too limited to compare statistically with crossbred mice, but there was a high frequency of the defect among those inbred mice on the deficient diets.

The incidence of the swimming defect for crossbred mice of experiment B was lower than in experiment A. All of these offspring were scored for the otolithic defects (table 3), and the incidence of mice with some morphological defect was somewhat higher than the swimming defect (13 to 20%, 8 to 18%, 20 to 37%, 35 to 46%

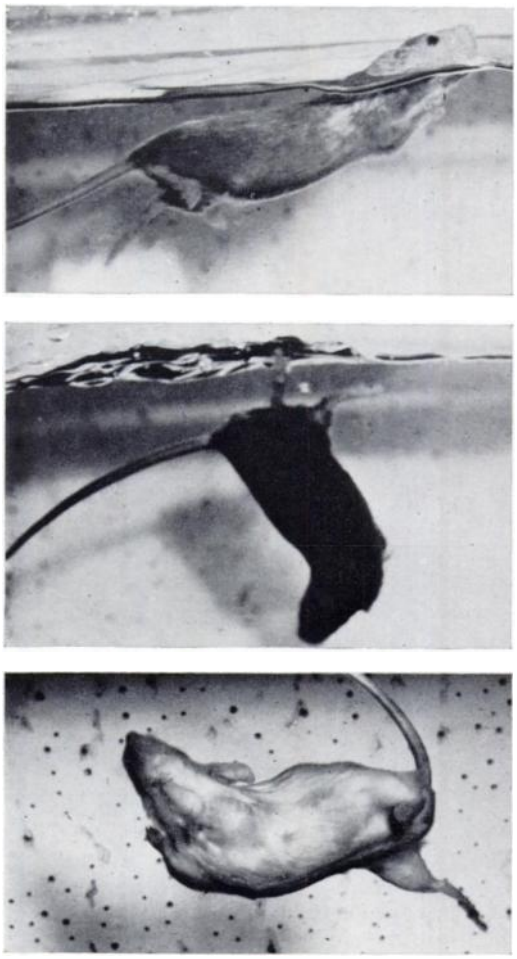

Fig. 1 Normal and ataxic mice tested for swimming ability. A normal mouse (top) does not spontaneously lower its head beneath the surface of the water. Many of the manganese-deficient mice (middle, black mouse and bottom, albino mouse) were unable to maintain balance and could not avoid swimming beneath the surface of water.

and 36 to 54%). The mean otolithic score was significantly different in successive litters and significantly lower for 1 ppm than for 3 ppm.

When animals were reared on the manganese-deficient diet (1 ppm, experiment E) they produced first litters, which as newborn animals exhibited a very much higher incidence of the morphological defects than any previous experiment had indicated. Three hundred offspring of first litters were produced in this manner (table 3) of which nearly 80% exhibited some otolithic defect. The mean otolithic score was 52 ± 4% for first litters, significantly lower than any other value obtained for third litters on previous feeding regimes.

There is one interesting exception to the increased incidence of otolithic defects with prolonged feeding of the manganese-deficient diet (experiment D). Mice, fed for six generations the diet containing 3 ppm manganese, exhibited a very significantly reduced incidence of the swimming defect. Whereas the incidence in the second generation was 83%, in the sixth generation it was 6% (table 2).

Morphological observations. Preparations of the intact otic capsules indicated that the only significant effects of manganese deficiency on the inner ear involved the formation of otoliths (fig. 2). There was considerable asymmetry in the degree of otolith formation within litters, within individuals and also within the same ear. Bilateral asymmetry was common, but there was no apparent direction to the left-right asymmetry. However, asymmetry within the same ear always indicated that the utricular otolith was as severely affected as, or more severely affected than, the saccular otolith.

Staining with toluidine blue revealed that in normal animals the otolithic matrix, in which the otolithic crystals were embedded, stained metachromatically, indicating the presence of acid mucopolysaccharides. No such matrix could be seen in those ears from manganese-deficient mice which also lacked the otolithic crystals.

Correlation of behavior and otolith defects. For simplicity all of the otolith scores of 1 to 3 were reduced to "+," and individual mice were categorized without regard to whether the left or right ear exhibited the defect (table 4). Without exception, the mice whose ears were morphologically "normal," (i.e., had some otolithic crystals in all four otoliths) swam normally; mice which lacked all four otoliths were severely affected. There were three other categories of otolith development; some of these animals behaved normally, and others were severely affected. A few mice were scored as only partially affected because they managed to stay afloat, although exhibiting considerable instability.

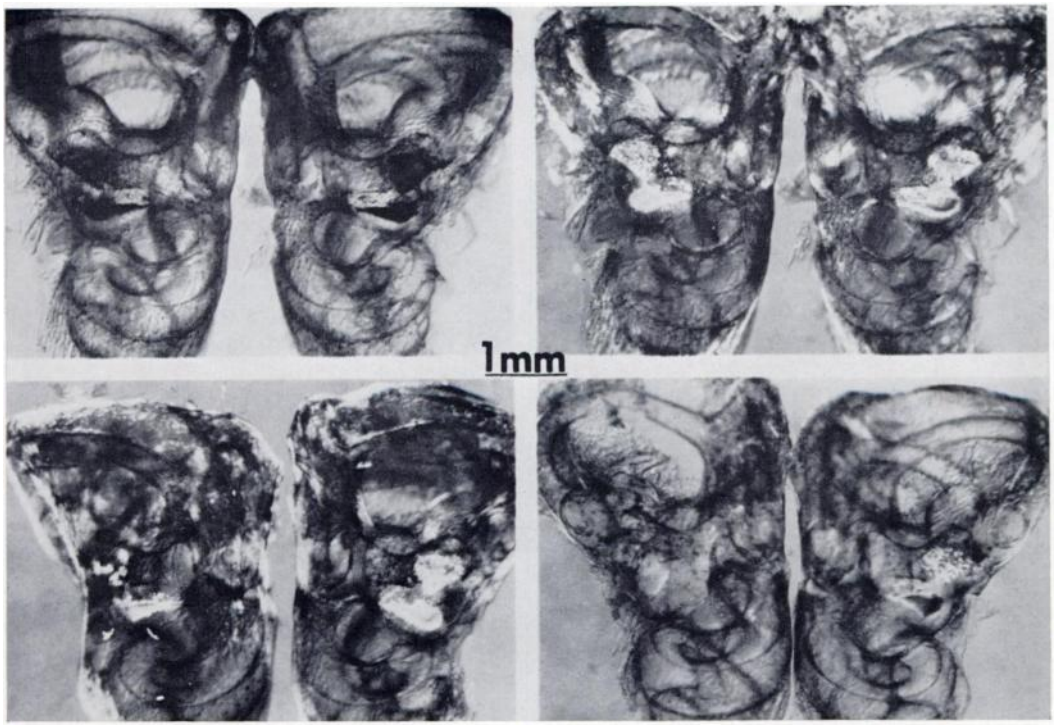

Fig. 2 Cleared otic capsules from normal and manganese-deficient adult mice. The labyrinth, including semicircular canals, cochlea, utricle and saccule, is visible in each photograph. The pigmented utricle is a horizontally oriented, cylindrical chamber which connects the canals. The saccule is beneath the utricle and at an angle to it. The otoliths within the utricle and saccule appear dark in transmitted light (top left) but are white in other photographs made with polarized light. The two top photographs are made of the same pair of normal ears. The lower left photograph exhibits reduction of left utricular otolith, and the lower right photograph has no otoliths in the left ear, whereas they are normal in the right ear.

TABLE 4

Correlation between state of otolith formation and ability to swim in control and manganese-deficient mice [1]

	State of otolith formation		Ability to swim
	u/s	u/s [2]	
1.	+/+	+/+	684 Normal: none affected
2.	0/+	+/+	5 Normal: 1 partially affected
3.	0/0	+/+	26 Normal: 9 partially affected: 7 severely affected
4.	0/0	0/+	2 Normal: 4 severely affected
5.	0/0	0/0	None normal: 107 severely affected

[1] All mice in experiments A, B, C, and D for which both swimming and otolith scores were available.
[2] The code: u = utricular otolith of either ear, s = saccular otolith of either ear, + = any otolithic crystals present, O = no otolithic crystals present. For example, no. 2 indicates that only one utricular otolith was absent, the others being normal or nearly so; no. 3 indicates that both the utricular and saccular otoliths of one ear were absent whereas those of the other ear were normal, or nearly so.

In addition to the swimming defect, some of the animals also exhibited head tilting and abnormal responses when they were held by the tail. Head retraction and ataxia were exaggerated when the mice were placed in the water and frequently caused the animal to swim in a backward somersault manner beneath the surface of the water. The head ataxia was exaggerated immediately after the animal was re-

moved from the water and allowed to regain its orientation on a solid surface. Although these patterns were less diagnostic than the swimming criterion, they were, nevertheless, frequently observed among the manganese-deficient mice. Whereas the direction of the head tilting was constant for a given individual, it did not appear to be strictly correlated with the morphological asymmetry of the otoliths. Head tilting was, however, an almost certain indication that the animal had some otolith defect and was unable to swim normally.

Manganese supplementation. The mice used in experiment E, fed 1 ppm manganese, had produced first litters with a very high incidence of otolithic defects (table 3). After determining matings, these females were fed the supplemented diet (1,000 ppm Mn) beginning at days 10 to 15 of gestation (table 5). The results indicate that supplementation on day 15 produced no significant difference in otolith development from that observed in unsupplemented animals. Supplementation beginning on days 12 to 14 yielded only intermediate degrees of otolith formation. Only when supplementation was initiated as early as day 11 was there a complete and normal pattern of otolith development.

In addition to the intermediate response obtained by manganese supplementation starting on days 12 to 14, there were strikingly abnormal effects upon the location of the crystals. The crystals were often scattered throughout other portions of the labyrinth, including the canals, ampullae and cochlea (fig. 3). In a few cases, there were also exceptions to the previously stated rule that the utricular otolith was more severely affected than the saccular otolith.

Manganese-deficient chicks. Ataxia in chicks was observed in early studies of manganese deficiency, but the basis of the ataxia was undetermined (17, 18). The ataxic chick frequently retracted its head to the point of nearly resting it on its back. It exhibited much difficulty in maintaining balance during movements. Birds have utricular and saccular otoliths together with a third, or lagenar, otolith which is located at the end of a long, slightly curved cochlea, or lagena. Most of a dozen or more of the manganese-deficient chicks exhibited defects in some or all of the otoliths. The defects were similar to those seen in mice and often involved the presence of abnormally large crystals. Such crystals were often misplaced toward the ampullae (fig. 4).

DISCUSSION

The results presented here indicate that manganese deficiency during embryonic development can cause specific morphological and behavioral abnormalities. The primary defect concerned involves the formation of otoliths within the membranous labyrinth. Failure of otolith development may be related to abnormal mucopolysaccharide synthesis, such as has been found in bone (14–16). Other studies, in fact,

TABLE 5

Timed manganese supplementation (1,000 ppm) of manganese-deficient females during gestation (expt. E)

Day of beginning supplement [1] (fetal age)	Litters	Progeny	M.O.S.[2]	SD [3]
day	*No.*	*No.*	*%*	*%*
Unsupplemented	5	26	19	12
15	4	23	10	9
14	4	25	57	12
13	4	26	71	7
12	3	16	53	3
11	1	6	100	—
10	2	14	100	—

[1] Pregnant females were transferred from the purified diet containing 1 ppm Mn to one containing 1,000 ppm, beginning on the indicated day after observation of vaginal plugs, and were continued thereafter on the supplemented diet.

[2] M.O.S. = Mean otolith score calculated as the percentage:

$$\frac{\text{Otolith score observed per litter}}{\text{otolith score expected per normal litter}} \times 100$$

[3] SD = Standard deviation of mean otolith scores for the number of litters scored.

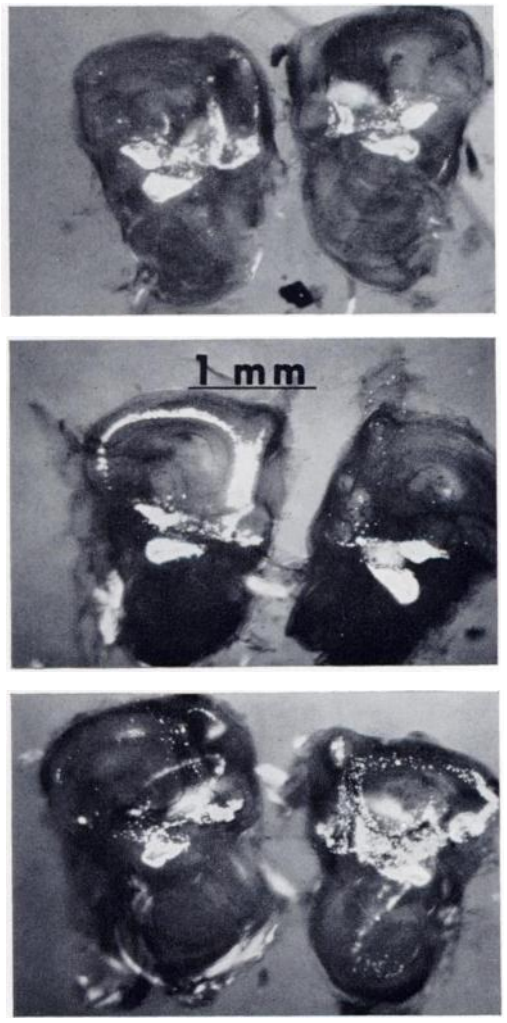

Fig. 3 Cleared otic capsules from newborn, manganese-deficient mice supplemented between days 12 and 14 of gestation. These are cleared preparations of the intact otic capsules comparable to the adult ears in figure 2. There is extensive deposition of otolithic crystals throughout the extramacular portions of the labyrinth, including the semicircular canals, ampullae and base of the cochlea.

indicate that manganese is probably essential for mucopolysaccharide synthesis in both cartilage and otolith matrix (31).

The simplest assumption is that manganese is a cofactor for some enzyme directly or indirectly involved in the synthesis of mucopolysaccharides. However, the fact that manganese is required as early as day 11 of gestation in the mouse, and that matrix and crystals appear only between days 15 and 16 (29) raise an intriguing question regarding the biochemical role of manganese in these processes. Moreover, it remains to be demonstrated how the mucopolysaccharide matrix is a prerequisite for normal otolith formation.

It is apparent from the present experiments and from the comparable genetic studies,[7] that the process of development of otoliths is especially sensitive to lack of manganese and requires the element at critical times. Manganese deficiency followed by supplementation has demonstrated that it is possible to disrupt the normal pattern of development so that crystalline material was abnormally deposited throughout the membranous labyrinth. However, this misplacement of crystals may be an exaggeration of a normal transitory phenomenon in otolith development; [8] such abnormally located crystals were not found later in development. These induced disturbances may enable us to separate the processes of matrix synthesis, secretion of calcium carbonate in the endolymph and deposition of crystals on the matrix.

The otolithic defects induced by manganese deficiency are indistinguishable from those associated with several mutations (*pallid*, *muted*, and *mocha* in mice; [9] *gray-loco* in chukar-partridges; [10] and *ocular albinism* in rabbits [11] (32). Manganese supplementation of *pallid* mice reduced the otolithic defect [12] (24), and it has a probable remedial effect on the *gray-loco* mutants of chukar-partridges.[13] It is, therefore, possible that such genetic anomalies and specific nutrient requirements may be more common in other animals than was heretofore suspected. It is postulated, for example, that the sex-linked form of *ocular albinism* in man (33) may also involve in-

[7] See footnote 6.
[8] See footnote 6.
[9] *Muted* has been described by M. F. Lyon in the Mouse News Letter (July, 1965). *Mocha* has been observed by D. S. Deol and P. Lane, personal communication.
[10] *Gray-loco* mutants of the chukar-partridge were provided by Professors U. K. Abbott and H. Abplanalp, Department of Poultry Husbandry, University of California, Davis. Mr. Robert Craig has described more extensively the otolith defects in an M. S. thesis, 1969, University of California, Davis.
[11] Dr. Karin Magnussen, Bremen, Germany, has described *ocular* albinism in rabbits (32) and has provided a few specimens which were examined for labyrinthine defects.
[12] See footnote 6.
[13] See footnote 10.

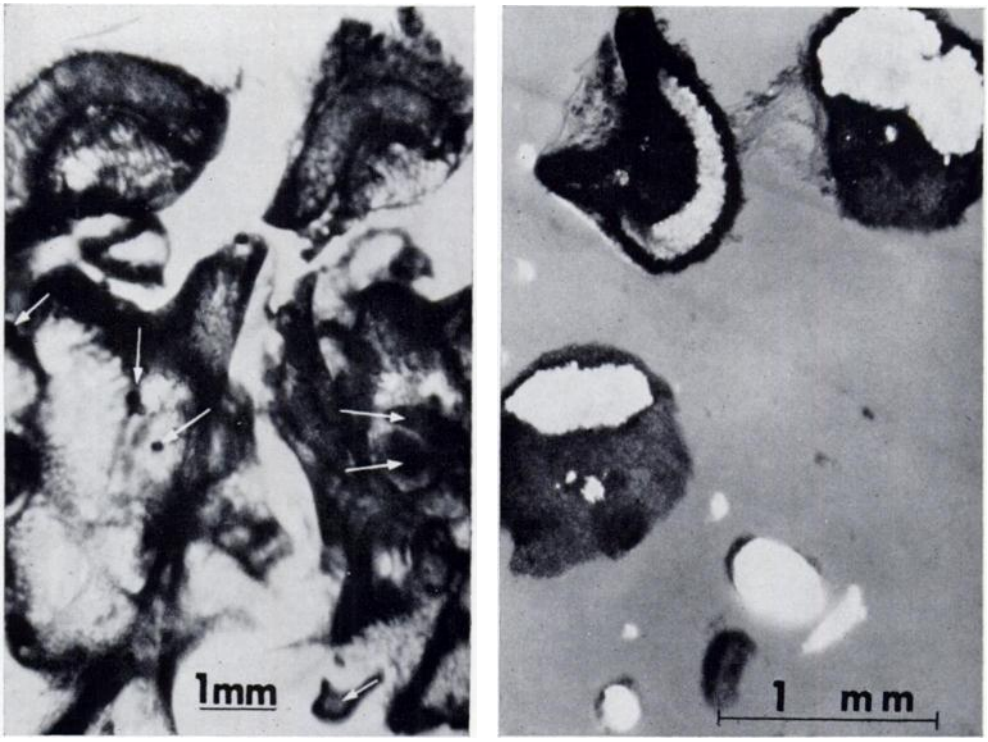

Fig. 4 Otic capsules and otoliths from normal and manganese-deficient chicks. The otic capsules (left photograph) were cleared and stained with alizarin red. Each capsule exhibits a portion of the superior semicircular canal, the unstained portion being due to the incomplete calcification at this stage in development. The normal ear (right capsule) exhibits three dense otoliths (indicated by arrows) within the utricle, saccule and lagena, top to bottom, respectively. The manganese-deficient ear (left capsule) exhibits only a few small crystalline foci (indicated by arrows), and some of these are located at extramacular positions. The dissected otoliths (right photograph) illustrate the shape and distribution of otolithic crystals. The three large otoliths (top of photograph) are from normal ear. The two small, white, ovoid masses represent foci from the manganese-deficient ear.

ner ear pigmentation and otolith development.[14] Thus, it may be possible to prevent the behavioral anomalies associated with this human defect, in a manner similar to that described for *pallid* mice.

These otolithic defects may be of more than developmental and anatomical interest. There is still uncertainty about the physiology and interrelationships of otoliths and cristae of the semicircular canals. Because of the specificity of manganese deficiency and of these particular mutations for otolith development, it may be possible to distinguish some aspects of labyrinthine function. The role of otoliths in eye nystagmus appears to be a very intriguing aspect which might be studied in animals exhibiting variability in otolith formation. Already our casual observation of some of the *tilted head*[15] mutant mice indicates that they may exhibit a rather unique ability to assume a vertical position in water, presumably because they possess abnormal otoliths present only within the sacculi.

Birds have a third, or lagenar, otolith as do most lower vertebrates. To our knowledge, no one has demonstrated its separate adaptive function. However, a careful physiological study of the behavior of manganese-deficient, ataxic birds, or of the *gray-loco* mutant chukar-partridges, might enable one to differentiate between the function of these three, differently oriented otoliths.

One of the most interesting aspects of these studies has been the indication of ge-

[14] See footnote 6.
[15] See footnote 6.

netically determined differences in minimal requirements for manganese. There are several reports of strain differences in poultry which imply genetic differences of this sort (34–37). It has generally been stated that it is the heavier breeds (New Hampshire Reds, and Barred Plymouth Rocks) which require more manganese than a lighter breed (White Leghorn). The difference in body size may or may not have a causal relationship to manganese requirements. However, the above-cited examples of pigment mutations with otolithic defects, as well as additional evidence relating pigmentation and trace elements,[16] lead to the suggestion that difference in pigmentation of poultry breeds may be an important factor in manganese requirements. To investigate this possibility, pigmented and nonpigmented substrains of the same breed might be tested for minimal manganese requirements, or for incidence of ataxia and otolithic defects on a deficient diet.

Our own findings in mice indicate a possible genetic difference, other than that demonstrated for *pallid* mice, for manganese requirements. The first evidence of this possibility was obtained from the data of table 2. It was found that the incidence of the otolithic defect decreased from 83% to 6% between the second and sixth generations of feeding crossbred mice the 3 ppm manganese diet. It is our interpretation that natural selection for ability to survive and reproduce on this diet may have occurred. At best, this evidence indicates that it would be worthwhile to perform appropriately designed selection experiments for ability to utilize manganese.

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[16] See footnote 6.

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