Studies on Nutritional Factors in Mammalian Development[1]

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It is a special pleasure for me today to be able to contribute some small part to the honoring of my professor, Dr. Agnes Fay Morgan, who has had a most important influence on my life and continues to be a source of inspiration.

Much of the work I shall discuss saw its beginnings in the investigations of distinguished researchers who are present today, both as speakers and as members of the audience. This example of the continuity of our science provides a fitting tribute to our guest of honor and to her dedication to the future of nutrition research.

My subject, the role of nutritional factors in mammalian embryonic development, fits appropriately in the context of the theme of this symposium, because it is during the time-span being discussed today that research on this subject began. Systematic studies of the influence of nutrition on the prenatal development of mammals can be said to have begun with the observations of Hale about 1935 (1).

Before this time, a considerable number of experiments had been carried out with avian and amphibian eggs, and it was recognized that environmental factors could influence the development of these embryos (2, 3). Yet even in these species, little thought was given to the role of nutrition in embryonic development, and as far as mammalian embryos were concerned, genetics claimed the day. It was thought that the mammalian embryo was too well-protected by the maternal organism to be affected by environmental influences, and congenital abnormalities, that is, abnormalities existing at birth, were generally considered to be hereditary (4).

The first experimental evidence that a change in the environment could disrupt the normal development of a mammal appeared about 1935 when Hale, working in Texas, reported that pigs born to vitamin A-deficient sows had malformations including cleft lips and missing eyes. Hale (1) provided convincing evidence that the malformations were not hereditary. Thus, the first clear proof that an environmental factor could produce congenital malformations came from nutrition research.

Hale's experiments provided valid, although accidental, proof of the influence of a nutritional deficiency on development. The work of Warkany and his colleagues, however, was the first experimental use of nutritional deficiencies in a deliberate attempt to study their effects upon the development of mammalian embryos. In the early '40's, Warkany published a series of papers reporting the production of congenital malformations in rats when the maternal diet was deficient in riboflavin. These malformations consisted almost entirely of skeletal anomalies, including shortening of the lower jaw, fusion of the ribs, syndactyly, and cleft palate (5).

Important contributions to this subject were made by a former associate of Dr. Morgan's, the late Dr. Marjorie M. Nelson, whose premature death two and one-half years ago was a great loss. Dr. Nelson was the first to use antimetabolites as a means

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of inducing nutritional deficiencies very severely and acutely in order to study their effects upon embryonic development. The effects of folic acid deficiency, as produced by x-methyl pteroylglutamic acid were extensively and systematically studied in this way (6–10). Dr. Nelson and her co-workers also studied pantothenic acid deficiency in pregnant rats, and found that various abnormalities, including exencephaly, urogenital anomalies, and necrosis of the paws resulted under these conditions (11).

And now I should like to discuss with you some of our own work. In this connection, I wish to acknowledge the valued collaboration of my colleagues and students, particularly Drs. C. W. Asling, Gladys Everson, and Paola Timiras.

One of the nutrients which we have studied in relation to fetal development is pantothenic acid. I had been interested in this vitamin since the days of my dissertation research as a student of Dr. Morgan's. Since Nelson and also Giroud of Paris (12) had shown that congenital malformations occurred in offspring of pantothenic acid-deficient rats, I was interested in investigating the biochemical relationships between pantothenic acid and coenzyme A during development, as well as the influence of a deficiency of the vitamin upon the offspring.

The rat fetus, however, because of its small size, is a difficult animal to work with for chemical studies. We therefore used the guinea pig. In the first aspect of the study, the changes in the levels of free pantothenic acid, bound pantothenic acid, and coenzyme A in the liver were determined during fetal and post-natal development. The results of this study are summarized in figure 1.

The concentrations of these compounds remained almost stationary from the 33rd to the 58th day of gestation. At 58 days of gestation, a sharp rise in bound pantothenic acid and in coenzyme A occurred which reached its maximum at 4 days after birth. These results suggested that the critical period with respect to pantothenic acid and coenzyme A in the developing guinea pig is in the period shortly before and after birth (13).

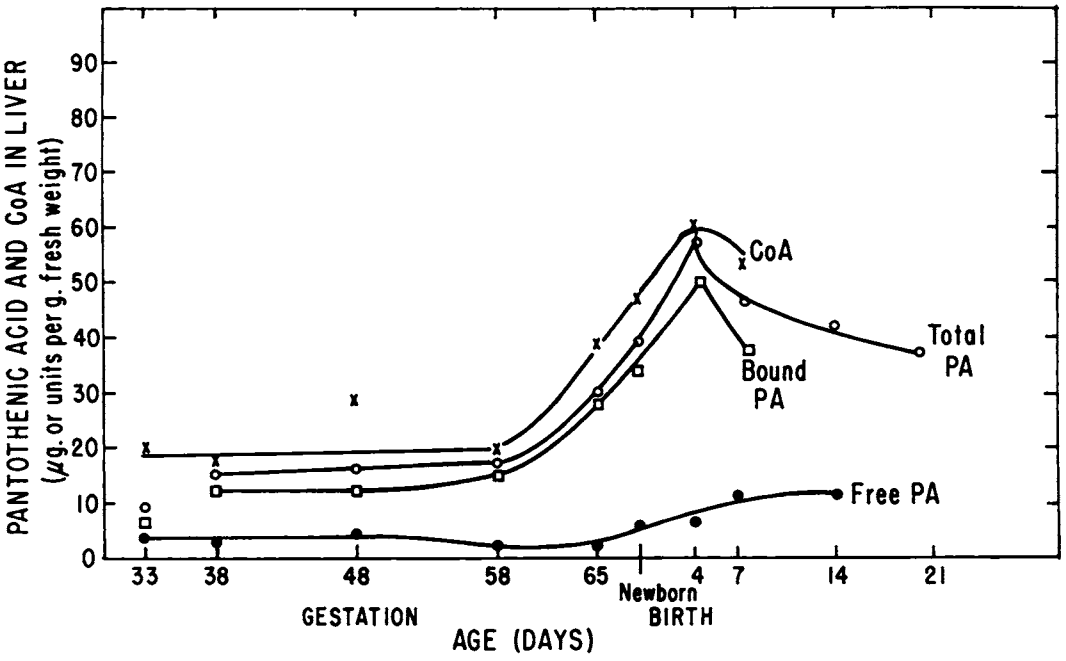

Fig. 1 Pantothenic acid and coenzyme A in livers of developing guinea pigs. Each point represents the mean of from 2 to 9 fetuses or young guinea pigs, in most cases, 5. (Hurley, L. S., and N. Volkert. Biochim. Biophys. Acta, *104:* 372, 1965.)

In the next phase of this work, the effect of pantothenic acid deficiency during pregnancy upon the development of the offspring was studied (14). Preliminary experiments indicated that the pregnant guinea pig could not withstand long periods of a deficiency of this vitamin. A transitory deficiency period of one week was therefore chosen.

In this experiment, pregnant guinea pigs were transferred from a complete synthetic diet to the pantothenic acid-deficient diet for a period of one week during various periods of their pregnancies. One week of deficiency resulted in a reduction in the number of young born alive, and an increase in the number of litters aborted. This appeared to be especially severe when the deficiency period was the ninth week of gestation. (Gestation in the guinea pig is about 70 days, or 10 weeks.)

The effects of the transitory deficiency were also studied by measuring the levels of pantothenic acid and fat in the livers of the offspring (see fig. 2). The liver fat level of the normal newborn was found to be about 10 times higher than that of the adult, but by 7 days of age it had decreased almost to the adult value. In contrast, liver pantothenic acid concentration in the normal newborn was about 60% of the adult value, but rose during the first 7 days after birth.

A dietary deficiency of pantothenic acid during the 10th week of gestation appeared to produce a significant rise in the liver fat of the newborn. At the same time, the

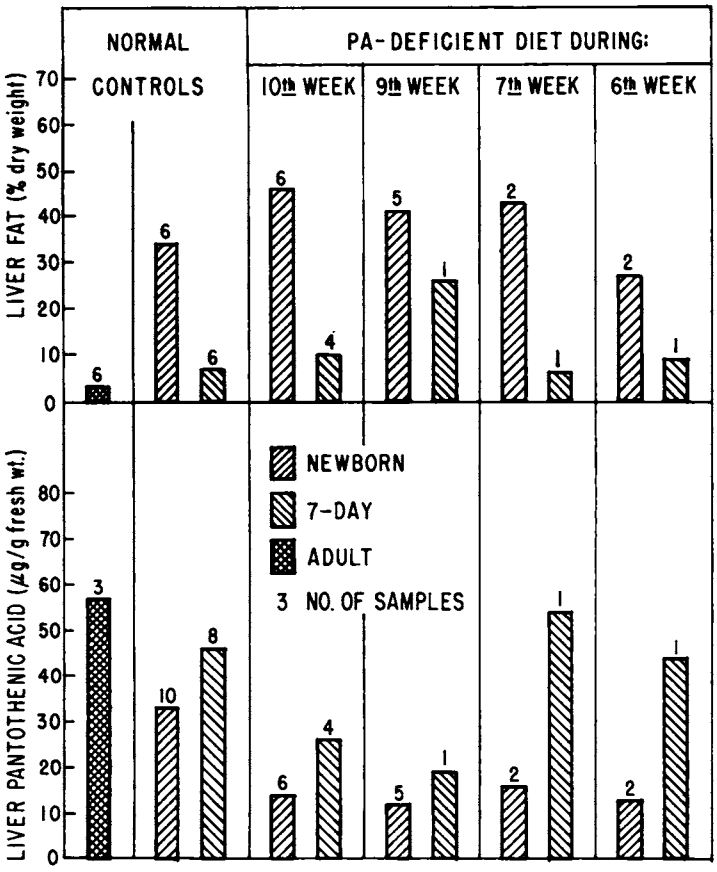

Fig. 2 Effect upon the offspring of a transitory deficiency of pantothenic acid during gestation in guinea pigs. Each bar represents the mean of several samples, the number of which is shown above the bars. (Hurley, L. S., N. E. Volkert and J. T. Eichner. J. Nutr., 86: 201, 1965.)

liver pantothenic acid level of this group was lower than normal, both at birth and at 7 days of age. Young whose mothers had received the deficient diet earlier than the 10th week showed no significant changes in liver fat, but did have low pantothenic acid levels.

The results of this experiment are in accord with our findings on changes in pantothenic acid and coenzyme A levels in fetal tissue. These works together suggest that the greatest need for pantothenic acid during fetal development of the guinea pig is in the period shortly before birth, possibly from about 58 days of gestation to term. It is of some interest that nutritional deprivation coming at the very end of pregnancy should have a demonstrable effect upon the offspring. This is in contrast with many experiments on teratogenic effects of nutritional factors, but this is not the only nutrient which has effects upon development relatively late in intrauterine life (15, 16).

Another nutrient with which we have been concerned is the trace element manganese. I am very pleased to describe this work today in the presence of Dr. McCollum, because of his early contributions to nutritional knowledge of this element. Orent and McCollum (17) reported in 1931 that the offspring of rats given a diet lacking in manganese were weak and did not survive. The early interpretation was that the deaths occurred because the females failed to nurse their young, and I understand that newspaper reports of the day hailed the discovery of "the mother-love factor." Daniels and Everson (18), however, showed that the high death rate was due to debility of the young themselves, since manganese-deficient mothers were capable of raising foster control young, although their own offspring died. Shils and McCollum in 1943 (19) showed further that the deficient offspring which survived exhibited an ataxia characterized by incoordination, lack of equilibrium, and retraction of the head.

It was actually known since 1939, when Dr. Leo Norris reported on his experiments with chickens, that a maternal dietary deficiency of manganese results in defective offspring which are ataxic (20). Various investigations were subsequently carried out in search of a lesion, either morphological or biochemical, which could account for this abnormal condition; but neither histological studies of the nervous system nor assays of various enzymes revealed the nature of the congenital defect resulting in ataxia (19, 21–23). It was to this question that we addressed ourselves.

Time does not permit me to present a detailed story. I can only attempt to show you a few of the parameters we have studied. Our experiments have been conducted with both rats and guinea pigs. In both species there is poor survival of the offspring of manganese-deficient females, and a high incidence of ataxia in the survivors (15, 24, 25). There is also a pronounced delay in the development of body-righting reflexes (26). These have been studied in two ways. In one test, the time required for the animal to turn from its back to its feet was measured; in the other, the ability of the animal to right itself in air was tested. In both tests, the manganese deficient young showed a marked delay in the development of reflexes responsible for body righting reactions.

The survival and the incidence of ataxia in the offspring were influenced by manganese supplementation during gestation. Table 1 shows that survival of manganese-deficient rats to 28 days of age was very low, only 11%, and 81% of these were ataxic. If, however, the mother was given the manganese-supplemented ration for only 24 hours on day 14 of gestation, ataxia was completely prevented. If supplementation was given on day 18, it had no effect at all on the ataxia, and this was true even if the control ration was continued from day 18 of gestation until weaning (15, 25). Thus, the congenital ataxia of manganese-deficient rats was due to an irreversible defect occurring between day 14 and day 18 of gestation.

The delayed development of the righting reflexes as well as the behavior of the animals led us to examine the vestibular portion of the inner ear. This was first done by examination of the skulls in alizarin-stained specimens. We found that

TABLE 1

Survival and incidence of ataxia in manganese-deficient young

Treatment	No. litters	Young		
		No. born alive	Survival to 28 days	Ataxia
			%	%
Mn-supplemented entire period	61	406	54	0
Mn-deficient entire period	30	191	11	81
Mn-supplemented only for 24 hours during gestation on:				
day 14	23	172	51	0
day 16	29	186	51	62
day 18	36	220	39	84

there was a marked delay in the ossification of the otic capsule, the bony covering surrounding the inner ear (27). In addition to the delayed development of ossification, there were actual malformations of this structure. Histological examination of the inner ear showed that there was a progressive degeneration of the neural epithelium of the semi-circular canals in the deficient rats. This degeneration was extreme by 4 days of age, but was already present at birth (28).

Thus, we were able to show that manganese deficiency during gestation in rats resulted in a defect of the inner ear consisting of gross malformations of the vestibular apparatus and histological damage to its neural epithelium. This defect would account for at least some of the abnormal symptoms of manganese-deficient offspring. Other manifestations of the deficiency, however, such as tremor, do not appear to be related to vestibular function. We were therefore interested in investigating another aspect of the nervous system.

In one experiment, the electroshock threshold, the amount of current in milliamperes required to produce convulsions, was measured (see table 2). By means of appropriate supplementation with manganese, the two variables of congenital ataxia and manganese deficiency were separated; this allowed the measurement of response to electroshock in manganese-supplemented and manganese-deficient rats, either with or without ataxia (15, 25). The threshold for convulsive seizures was significantly lower than normal in the two manganese-deficient groups, indicating that brain excitability or convulsability was increased in manganese-deficient rats regardless of the presence or absence of ataxia. Thus the level of manganese in the body appears to be important in determining the susceptibility of an animal to convulsive states (29).

Another system which is profoundly affected by manganese deficiency is the skeleton (see fig. 3). In deficient animals, there is disproportionate growth of the skeleton. This is already apparent at birth, and involves shortening of the long bones, and doming of the skull. There are also curvatures of radius and ulna, ulnar deviation of the forepaws, scoliosis and kyphosis (30–32). In addition, a disorder of the knee joint occurs in which there is abnormal development of the tibial epiphysis (33).

TABLE 2

Electroshock threshold in normal and manganese-deficient rats

Group	No. rats	EST[1]
		ma
1 Not deficient, not ataxic (Mn^+ controls)	14	19.50
2 Mn-deficient, not ataxic (supplemented day 14)	12	14.58[2]
3 Mn-deficient, ataxic (Mn^-)	13	16.42[2]
4 Not deficient, ataxic (supplemented day 18 and thereafter)	6	19.08

[1] Electroshock threshold in milliamperes.

[2] $P < 0.001$ as compared with Mn^+ control (Student's t test).

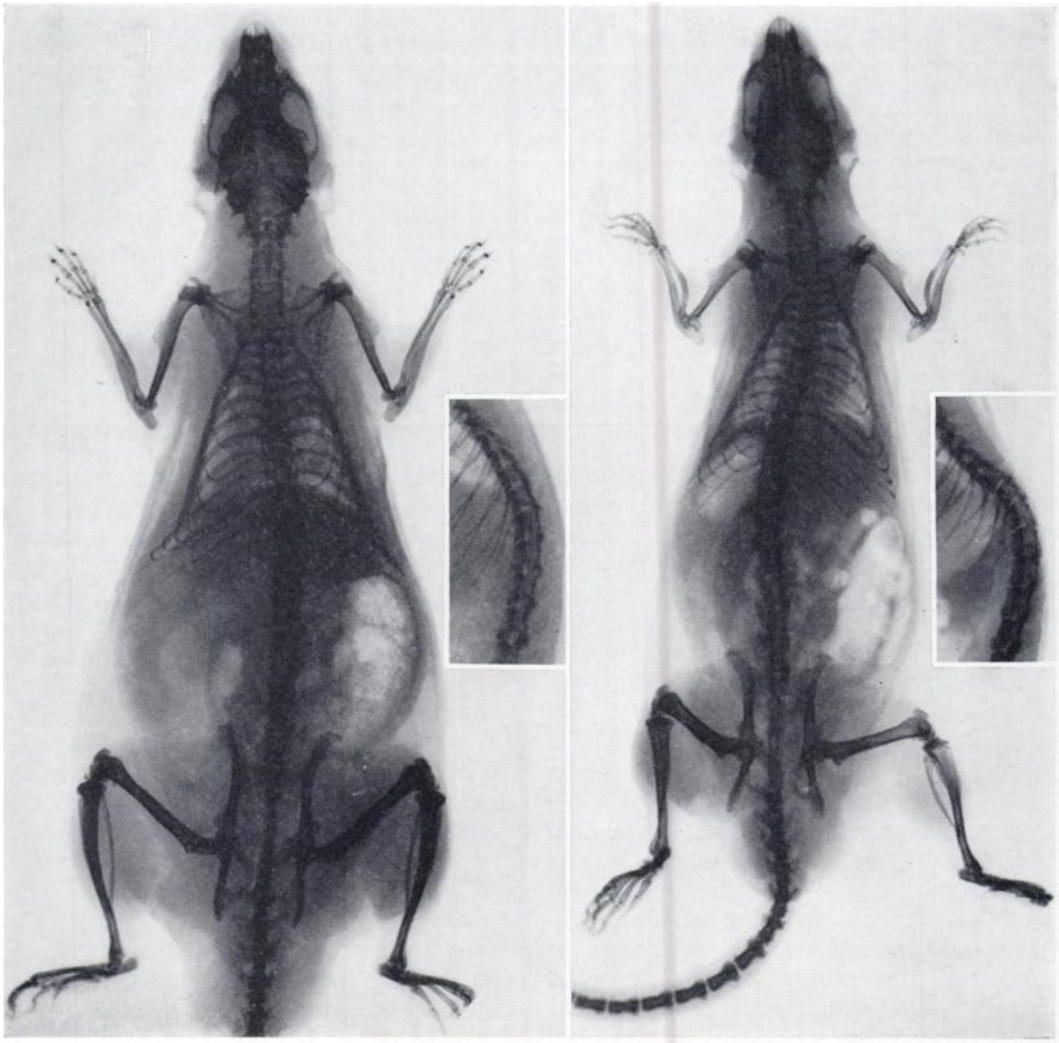

Fig. 3 Whole-body roentgenograms of 7-month-old manganese-supplemented and manganese-deficient rats. Note stunting of growth, curvatures of radius and ulna, ulnar deviation of forepaws, scoliosis, flared lower pelvis and thickening of upper portion of tibia in the deficient animal; in inserts (lateral view of thoracolumbar junction) note kyphosis. (Asling, C. W., and L. S. Hurley. Clin. Orthop. 27: 213, 1963.)

Various aspects of the skeletal as well as the vestibular abnormalities suggested that manganese played a role in cartilage metabolism. Analyses of mucopolysaccharide precursors substantiate this view (see table 3). In epiphyseal cartilage from manganese-deficient rats, the levels of hexuronic acids, glucosamine, and galactosamine were significantly reduced, both at birth and at 28 days of age. Similar effects were seen in guinea pigs (34).

Manganese-deficient animals also show abnormal electrocardiograms (35) and we have found abnormal oxidative phosphorylation in isolated liver mitochondria.[2]

Thus, there is a spectrum of abnormal changes resulting from a congenital deficiency of manganese. These include physiological changes, such as ataxia, abnormal

[2] Kagawa, Y. 1962 Some biochemical properties of mitochondria from normal and manganese-deficient rats. Master of Science Thesis, University of California, Davis.

TABLE 3

Mucopolysaccharide precursors in epiphyseal cartilage in rats

Age	Diet	No. rats	Hexuronic acids	Glucosamine	Galactosamine
			% dry wt	*% dry wt*	*% dry wt*
Newborn	Mn^+	10[1]	11.3	0.51	7.10
	Mn^-	10[1]	7.85[2]	0.45[2]	4.35[2]
28 Days	Mn^+	4	3.30	0.23	2.18
	Mn^-	5	2.37[2]	0.17[2]	1.26[2]

[1] In 2 pooled samples of 5 animals each.
[2] $P < 0.02$ as compared with Mn^+ Control (Student's t test).

brain function, and electrocardiogram abnormalities. There are also morphological changes, such as those seen in the inner ear, and skeletal abnormalities. And, finally, there are biochemical changes, such as decreased levels of mucopolysaccharide precursors, and disturbed oxidative phosphorylation in mitochondria.

The third and last nutrient which I would like to discuss is zinc. We became interested in zinc because of the similarity of some signs of zinc deficiency to those of manganese deficiency. In order to study the influence of zinc deficiency on embryonic development in rats, it was necessary first to establish that a severe deficiency state could be produced. This was accomplished by the use of a diet containing soybean protein, and by stringent elimination of sources of zinc contamination from the environment.

Under these conditions, animals receiving the zinc-free diet (0 ± 2 ppm by X-ray fluorescence analysis) from weaning showed almost no growth, and developed signs of severe zinc deficiency. These included alopecia, dermatitis, and a hunched, almost kangaroo-like posture (fig. 4). When these extremely abnormal animals were supplemented with zinc, their growth rate immediately rose and quickly reached that of the controls (fig. 5). In addition, all outward signs of zinc deficiency disappeared, and the animals became perfectly normal in appearance.

Under these extreme conditions, almost no reproduction was possible. The females showed severe disruption of the estrus

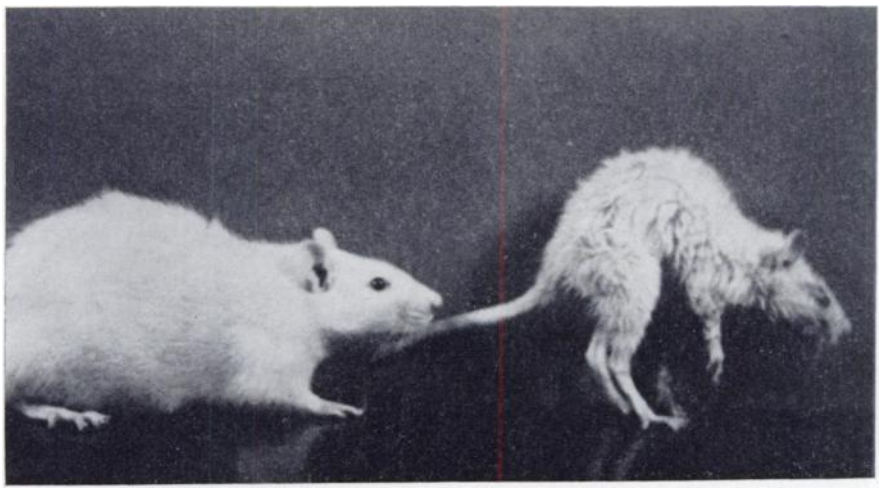

Fig. 4 Normal and zinc-deficient female rats after seven weeks on a purified diet containing either 40 or 0 ppm of zinc. Note ruffled hair, dermatitis, abnormal posture, and depressed growth in animal on right.

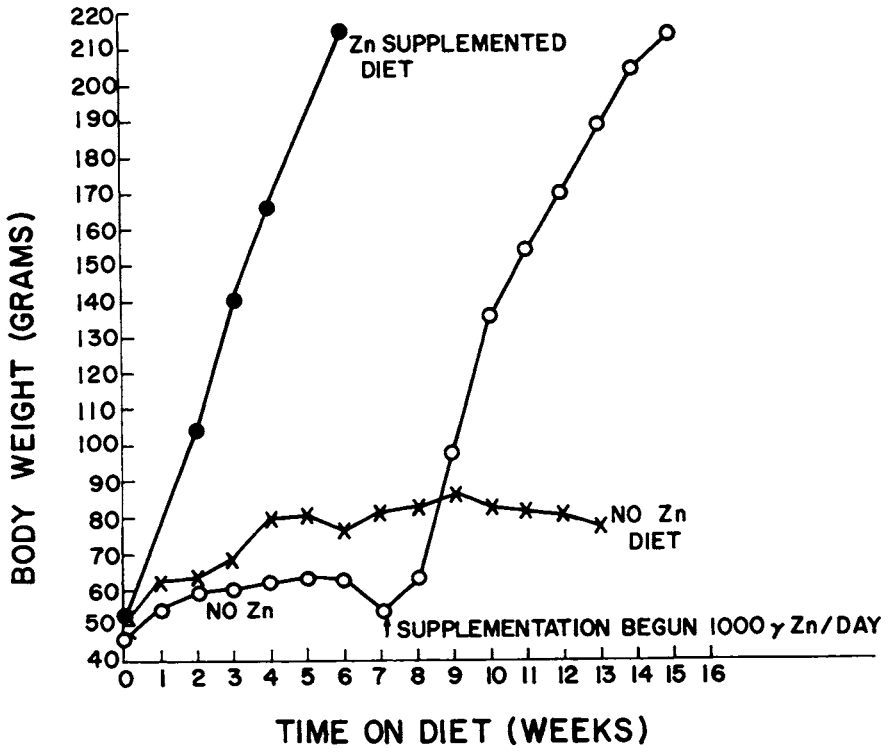

Fig. 5 Examples of growth curves of rats illustrating failure of growth in zinc-deficient animals, and rapid response of growth to oral supplementation with zinc.

cycles; in most cases no mating took place. Therefore, in order to study the effect of zinc deficiency on prenatal development, it was necessary to induce a less extreme form of deficiency which would permit reproduction to occur.

This was successfully accomplished by the procedure of maintaining the females on a marginally deficient diet until maturity. This diet did not produce signs of zinc deficiency in the animals. At maturity the rats were mated with normal males fed a stock ration, and were given either the extremely deficient ration containing no zinc, or the zinc-supplemented ration. In order that we could examine every living fetus, the animals were delivered by Caesarean section on day 21 of gestation, thus preventing the eating of defective young by the mother.

Table 4 shows that the rats which received the zinc-deficient diet lost rather than gained weight during pregnancy, and less than half of them had living young at term. The zinc-deficient females also had a smaller number of young per litter than did the controls, and the young were less than half the normal body weight. In addition, almost all of them, 98%, showed gross congenital malformations.

We also looked at all implantation sites in the uterus to have information on resorptions. Table 5 shows that in the deficient females, all but one of 129 implantation sites were affected. That is, the implantation site showed either a resorbed conceptus, or it had given rise to a malformed fetus. Thus, 99% of the implantation sites in deficient females were affected, as compared with 2 to 3% resorptions in the normal rats.

In figure 6, the appearance of these fetuses is shown. There is peculiar shaping of the head, clubbed feet, fused or missing digits, short lower jaw. Figure 7 shows an example of extreme syndactyly (fused or missing digits). Seventy-four percent of the deficient fetuses had this anomaly.

TABLE 4

Reproduction in zinc-deficient rats

No. rats	Net body wt change during gestation	Rats with living young (day 21)		Living young day 21				
				Total no.	Avg/litter	Body wt	Abnormal	
	g	No.	%			g	No.	%
				Stock diet				
5	+98	5	100	59	11.8	5.6	0	0
				Zn-supplemented controls				
12	+56	12	100	122	10.2	5.5	0	0
				Zn-deficient				
16	−19	7	44	43	6.1	2.1	42	98

TABLE 5

Implantation sites in zinc-deficient rats

Group	Implantation sites				
	Total no.	No. resorbed	No. abnormal fetuses	Total affected	
				No.	%
Stock	60	1	0	1	2
+Zn controls	126	4	0	4	3
Zn-deficient	129	86	42	128	99

Fig. 6 Fetuses at term from rats fed zinc-supplemented ration (on far left) or zinc-deficient ration. Note small size, abnormally shaped heads, clubbed feet, short lower jaw, and short or absent tail.

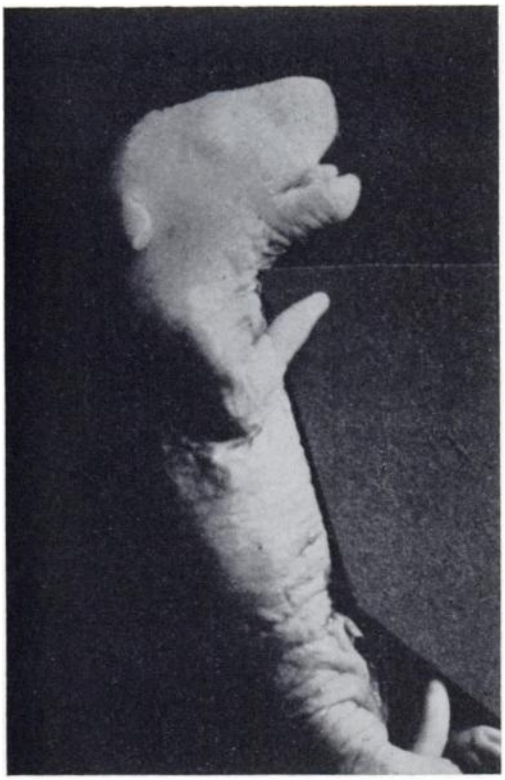

Fig. 7 Extreme syndactyly (fused or missing digits) in a fetus from a zinc-deficient rat on day 21 of pregnancy.

A striking proportion of the deficient offspring, 84%, had brain abnormalities including hydranencephaly. In this condition, there is enlargement of the ventricles, as well as a lack of development of the cerebral cortex.

The congenital malformations produced by zinc deficiency were varied and occurred in high incidence (table 6). A large number of skeletal defects were seen, in incidences ranging from 19% for short or missing lower jaw, to 81% for curly or stubby tail. A high incidence of soft tissue malformations were also seen, including hydrocephalus, small or missing eyes, hernias, and heart, lung, and urogenital abnormalities (36).[3] Since the anomalies occurred in such high incidence, I believe we have a good system in which to attempt an elucidation of the biochemical and enzymatic mechanisms by which these profound disruptions of normal embryonic development were brought about.

In conclusion, I have presented three examples of the interaction of nutrition and embryonic development. By chance, the three examples also illustrate three different aspects of the general study of this subject. In the case of pantothenic acid, we saw a manifestation of biochemical changes. In zinc deficiency, on the other hand, severe morphological alterations were obvious. With manganese deficiency, it was primarily physiological abnormalities which first attracted our attention.

The study of the role of nutritional (or indeed, any environmental) factors can be approached through any of these three avenues. To provide a meaningful explanation, however, in relation to modern concepts of biology, all three of these avenues must be brought together and the influence of nutritional factors on the embryo must be understood in terms of the correlation of morphological, physiological, and biochemical events in development.

TABLE 6

Types and incidence of gross congenital malformations in zinc-deficient fetuses[1]

Malformation	%
Cleft palate	26
Short or missing mandible	19
Scoliosis or kyphosis	70
Clubbed forefeet	49
Clubbed hindfeet	51
Fused or missing digits	74
Curly or stubby tail	81
Hydrocephalus or hydranencephalus	84
Small or missing eyes	44
Herniations	40
Heart abnormalities	26
Lung abnormalities	56
Urogenital abnormalities	46

[1] 43 fetuses examined.

[3] For data published since this review was prepared, see Hurley, L. S., and H. Swenerton, Proc. Soc. Exp. Biol. Med., *123*: 692, 1966.

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