EFFECT OF DIET DURING PREGNANCY UPON THE INCIDENCE OF CONGENITAL HEREDITARY DIAPHRAGMATIC HERNIA IN THE RAT

Failure to Produce Cystic Fibrosis of the Pancreas by Maternal Vitamin A Deficiency *

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The initial purpose of these experiments was to attempt to produce cystic fibrosis of the pancreas in newborn rats by means of breeding on a diet containing the minimum amount of vitamin A compatible with reproduction. The hypothesis that diet during pregnancy might play a rôle in the production of this lesion was based on three considerations which appeared valid when the experiments were begun in 1939. (1). Cystic fibrosis was known to occur in several siblings but no instance was known of its occurrence in more distant relatives.[1] Several such cases have since been observed by myself and others.[2] A disease of this pattern of incidence might be due to some untoward circumstance of pregnancy and is not necessarily of genetic origin. (2). The lesions found consist of obstruction of various epithelium-lined ducts, especially those of the pancreas. The obstruction is now believed to be the result of abnormalities in the material secreted.[3,4] (3). Obstruction of ducts by metaplastic epithelium had been described in experimental vitamin A deficiency.[5]

It seemed within the range of possibility and compatible with the facts then known, that the disease might be the result of vitamin A deficiency in the fetus. Since clinical vitamin A deficiency is not a characteristic of the mothers of patients with cystic fibrosis of the pancreas, a mechanism for the production of fetal deficiency without maternal deficiency must be postulated if this be the cause of the disease. Three explanations were devised: First, that an anomaly of maternal metabolism might lead to fetal deficiency; second, that the fetus, owing to a genetic abnormality, had an unusually high requirement of the vitamin; and third, that a degree of vitamin deficiency might exist which was adequate for the mother but inadequate for the fetus. Hence, either in the presence of a genetic metabolic anomaly or in normal animals, there might exist a level of vitamin A in the maternal diet which was compatible with maternal health and fetal deficiency.

The first step in testing this rather elaborate scheme was to demonstrate by animal experimentation that vitamin A deficiency during

* This investigation was supported by the Commonwealth Fund.
Received for publication, February 27, 1948.

fetal life leads to cystic fibrosis of the pancreas. The following experiments failed entirely in this objective. They have, however, provided convincing support of the more general hypothesis that a level of nutritional deficiency during pregnancy may occur in which the mothers show few or no signs of deficiency but the young die in the first day or two of life. As the experiment progressed it also became apparent that another important concept had been proved, namely, that the frequency of expression of a hereditary congenital malformation may be increased by deficiency in the maternal diet.

The literature on the production of congenital malformations by means of deficient diets during pregnancy has been reviewed recently by Warkany.[6] The malformations which have been described as the result of breeding on diets deficient in vitamin A include a variety of anomalies of the eye,[7-9] the teeth, [10] and malformations resulting from disproportionate growth of the skeleton and nervous system, including hydrocephalus [11] and blindness due to constriction of the optic nerve.[12] A preliminary report of the occurrence of diaphragmatic hernia in the young of vitamin A-deficient rats was made by me in 1941.[13] No data have been found relating to the effect of deficiency of specific nutritional substances during pregnancy on the incidence of malformations in the human being. Nonspecific nutritional deficiency leads to lowered fertility and increased infant mortality.[14-16]

Methods

Experimental Animals. The rats used in all but one experiment were of a colony which originated in 6 white rats which were purchased in 1929 and bred in the laboratory since that time without the addition of a new strain. Breeding has not been consistently by brother and sister matings. The rats used as breeders for the continuation of the colony have been selected on the basis of size, general condition, and freedom from infection of the first litters. Post-mortem examination of the lungs of all animals for evidence of infection has therefore been part of the routine of all experiments for the 8 years and over 3000 animals preceding the present study. This procedure has decreased the incidence of lung infections from about 50 per cent to something less than 10 per cent of the adult animals of the stock colony and has inadvertently provided evidence that diaphragmatic hernia of major degree was at least not common in the strain under routine breeding conditions. No instances of this anomaly were observed and if they had been common they could hardly have been overlooked.

The rats used in one experiment (no. VIII) were of the Long-Evans

strain. They were bred from 2 females and a male obtained in 1939. Breeding was not exclusively by brother-sister matings but care was taken to breed only within the strain. By chance the original rats were all black, although some of the young were hooded. Black rats were selected as breeders, so that the rats used in experiment VIII were black. This precaution was taken to minimize the chance of admixture with the white stock colony.

In order to ascertain fertility and also the incidence of resorption of the fetuses and the duration of pregnancy, all matings were carried out after a few days or weeks of examination of the vaginal smear. The rats were mated only when the smear was of the estrous type and they were examined on the following day for sperm or plug. After positive mating the male was removed from the cage and the female was weighed. She was weighed subsequently about twice a week. Infertility was nearly always the result of failure to have normal estrous cycles, the more deficient animals continuously showing cornified epithelium in the smears. Occasional resorptions were observed. The chief complication of pregnancy was delayed parturition, which has previously been reported in vitamin A-deficient rats.[17]

Diets. The stock diet used throughout was as follows:

Whole wheat flour	670 gm.
Casein	150 gm.
Dried milk	100 gm.
Calcium carbonate	15 gm.
Sodium chloride	10 gm.
Butter	50 gm.
Brewer's yeast	5 gm.
	1000 gm.

In this diet the main source of vitamin A was butter and the amount of the vitamin was somewhat variable. The batches of diet were made up weekly and kept in closed tins. This diet has been used routinely for the stock colony.

The vitamin A-deficient diet, diet 30, was as follows:

Casein, defatted	180 gm.
Cornstarch	570 gm.
Vegetable fat (crisco)	50 gm.
Wheat germ	50 gm.
Salt mixture	50 gm.
Brewer's yeast	100 gm.
Viosterol	15 drops
	1000 gm.

The casein was subjected to continuous extraction with hot 95 per cent ethyl alcohol for a total of 20 hours over a period of 3 days by the method described by Sperry.[18] After 3 to 6 hours of extraction the casein was removed from the extraction bag, ground, sieved, and replaced. After conclusion of the extraction it was dried in air, sieved, and stored in cans until use. In the preparation of the diet the dry ingredients were first mixed. The viosterol was worked thoroughly into the crisco and this was then worked into the dry materials. The diet was made up weekly. It was not assayed for vitamin content but this was apparently adequate except for vitamin A, since rats showed normal growth and reproduction for many generations when given diet 30 supplemented by vitamin A.

Haliver oil (Abbott) was used as the source of vitamin A. Various dilutions were made with Wesson oil, the calculations being based on the manufacturer's assay (1 gm. = 50,000 international units; 1 gm. = 40 drops; 1 drop = 1250 i.u.) The various dilutions used were given letters for convenience, with the following estimated content of vitamin A: X, 625 i.u. per drop; Z, 125; A, 25; B, 5; C, 1; D, 0.2; E, Wesson oil.

The supplement was given in amounts of 1 drop per day per rat and was fed three times a week by medicine dropper. Care was taken that only rats receiving the same supplement were kept in the same cage, but no attempt was made to prevent coprophagy.

Preliminary Experiments to Determine the Maximum Deficiency of Vitamin A Compatible with Reproduction

A series of experiments was carried out with small numbers of animals in order to determine the lowest dietary level of vitamin A compatible with fertility and the birth of young. In these experiments the factors to be considered were the age at which the rats were transferred from the stock diet to diet 30, the age at mating, the daily supplement of vitamin A, the length of time during which it was given, and the relation of this period to pregnancy and parturition.

Experiment I. At the age of 70 days, 12 female rats were transferred from the stock diet to diet 30. At 90 days they were divided into five groups and these were given supplements A, B, C, D, and E respectively. The rats were mated and all bore litters at ages between 113 and 142 days. Three litters were missing on the day after birth (on B, C, and D levels). The rest showed no abnormalities except for a lower average weight at weaning in the rats bred on the lower supplements. It was concluded that the rats were started on the diet too late to be adequately depleted of vitamin A.

Experiment II. Nineteen female rats were started on diet 30 at the age of 35 days and the supplements Z, A, B, C, and E were started at the age of 60 days, each level of supplement being given to 3 of 4 rats. Mating was begun at 80 days. Estrous cycles were irregular and fertility

was subnormal. However, 11 of the 12 rats receiving Z, A, or B levels became pregnant. None of the rats receiving C or E supplements were successfully mated. After positive mating the supplement was discontinued.

The 54 young of the 7 rats receiving the supplements A or Z appeared normal on the day of birth but disappeared one at a time during the first week, so that only 10 survived to the age of weaning (21 days). No external abnormalities were noted, and few of the young were autopsied since only fragments of them were found. Of the 4 rats on the B supplement, 2 died during parturition and the other 2 gave birth. One litter was born during the night and had been eaten by morning. The other litter was dead when found; *3 of its 7 members were found to have hernias of the right side of the diaphragm* and no abnormalities were found in the other 4.

From this experiment the conclusion was drawn that the initial depletion of vitamin stores was adequate and that the supplements at the Z, A, and B levels were sufficient to permit positive mating but that the total deficiency at the end of pregnancy in rats receiving B supplement was too great for survival of the young, and insufficient vitamin was stored at the A and Z levels of intake to provide for the demands of pregnancy and lactation when the vitamin was withheld after positive mating. The occurrence of one litter with 3 rats having diaphragmatic hernia was considered a chance finding.

Experiment III. It was apparent that the deficiency was not great enough for the production of abnormal young in experiment I and too great for normal parturition and survival of the young in experiment II. Experiment II was repeated, using the surviving young of the rats of Experiment I, to evaluate the rôle of the diet of the previous generation. The results were essentially similar to those of experiment II. The chief contribution of this experiment was the finding of *right diaphragmatic hernia in 3 of the 17 surviving young rats.*

Experiment IV. Experiment IV was designed to discover whether the neonatal death of the young in the previous experiments was due to inadequate lactation and also whether supplement given late in pregnancy would increase the proportion of young surviving. The breeding mothers were composed of three groups: *a.* One group was placed on diet 30 at 35 days and mating was begun at 70 days; supplement B was begun on the 60th day and continued through pregnancy. *b.* A second group was placed on diet 30 at 30 days and given no supplement until the 16th to 21st day after successful mating when they received an initial dose of 4 drops of "Z" followed by 2 drops on alter-

nate days. *c*. A third group used as controls was kept and bred on the stock diet. The plan was to breed one of the control rats on the day following each positive mating of one of the deficient animals, and exchange half of each litter at birth or the following day. The control young were marked by clipping the end of the fifth toe of the right fore-

TABLE I

Experiment IV. The Results of Exchanging Half of Each Litter of the Vitamin A-Deficient Rats with Half of Each Litter of Controls, to Determine the Rôle of Lactation in the Neonatal Mortality.

Experiment	Nursed by rat no.	Young of rat no.	No. of young	Mean wt., gm., at		
				1–3 days	14 days	21 days
IV–a	3618 Deficient	3618	3 (1 dead at 8 days)	6 (2 days)	17.5	24.5
		3601	4	7.25	18.25	21.75
	3601 Control	3618	2	6.0	26.5	37.5
		3601	5 (1 dead at 8 days)	8.8	27.75	39.5
IV–a	3639 Deficient	3639	4	5.25 (1 day)	Dead by	
		3644	4	5.25		
	3644 Control	3639	4	5.25	4th day	
		3644	4	5.25	26.25	44
IV–b	3669 Deficient	3669	3 (1 dead at 2 days)	6.0 (3 days)	17.0	32.0
		3660	4	7.5	18.75	36.0
	3660 Control	3669	3	7.3	25.0	44.3
		3660	4	9.0	27.0	49.0
IV–b	3696 Deficient	3696	1	Dead (2 days)		
		3661	4	10	28.5	49
	3661 Control	3696	3	Dead (2 days)		
		3661	4	10		45

foot. Each control mother therefore suckled half the litter of the experimental animal and half of her own litter, and each experimental mother likewise had a mixed litter. When necessary, young rats were discarded so as to have no more than 8 in each litter.

This plan contained too many hazards and was successfully executed in only 2 cases of the *a* and 2 of the *b* groups, a total of 4 successes in 14 rats started on the experiment (Table I). Of the remainder, one proved sterile, 2 resorbed their litters, success in obtaining parallel control litters was lacking in 2, and the remaining 5 litters were born dead.

In the four experiments in which the exchange of litters was safely made, the results were varied. In the first, both litters were weaned, with obviously better growth in the litter cared for by the control mother. In the second, the entire mixed litter cared for by the experi-

mental animal and also her young cared for by the control mother died within a few days. The results in the third pair of rats paralleled those of the first, and in the fourth all of the offspring of the experimental mother died by the second day while all those of the control mother did well, whichever mother cared for them. Thus an example of each possible result was observed. The answer to the question whether

TABLE II

Experiment IV. Incidence of Diaphragmatic Hernia in the Young of Rats Bred on Minimal Amounts of Vitamin A Compatible with Reproduction. The Rats Were Placed on Diet 30 at 35 Days of Age. Group a Received Supplement B from the 60th Day, Group b Received Supplement Z Beginning at the 16-19 Day of Pregnancy.

	Rat	Z given on day	Number of offspring			
			Born	Survived to 21st day	Autopsied	With D.H.
Group a	3618	—	7	4	7	1
	3639	—	8	0	1	0
	3641	—	4	0	3	1
	3621	—	7	0	7	2
	3626	—	6	0 (4 killed)	4	1
	3645	—	8	6	6	0
	3628	(resorption)				
Total			40	10	28	5
Group b	3669	16	7	6	5	0
	3696	18	7	0	3	1
	3654	19	5	0	5	5
	3658	19	9	0	9	9
	3694	18	12	0	12	0
	3664	Resorption				
	3665	No successful mating				
Total			40	6	34	15
Grand Total			80	16	62	20

neonatal death was due to deficient lactation was not decisive, but observation of the young led to the belief that the majority of the young of the deficient mothers were too feeble to suckle.

Although the objective of this experiment was not attained, in the course of the careful watch necessary to carry out the experiment, for the first time a large percentage of the young were rescued from maternal ingestion. In the course of post-mortem examination of the young of the experimental animals an important observation was made. *Hernia of the diaphragm was found in a total of 20 of the 61 animals examined,* most of them from litters of the *b* group in which supplement was withheld until late in pregnancy (Table II). All of the young of 2 litters, numbering 5 and 9 respectively, showed a hernia of the diaphragm of severe degree.

Experiments Directed at Diaphragmatic Hernia

Experiment V. The first move in experiment V was to attempt to reproduce the lesions observed in experiment IV-*b*. This and subsequent experiments were based on the following standard procedure. The rats were taken from the stock colony and placed on diet 30 at the age of 35 to 42 days. Beginning at about the 70th day a vaginal smear was examined each day. When the smear was of the estrous type the rat was left overnight with a normal male which had been bred on an adequate diet. On the following morning a vaginal smear was examined for sperm or a plug. The male was then removed. The female was weighed on this day and twice a week thereafter. On the 16th to 18th day following the day on which the male was placed in the cage, the female was weighed and if there had been enough gain in weight to indicate pregnancy, the rat was given 4 or more drops of Z supplement by medicine dropper (500 i.u. of vitamin A) and 2 drops every 2 days thereafter. When mating was unsuccessful and vaginal smears showed a continuous production of cornified epithelium without estrus, 2 drops of Z supplement sometimes were given, and this often sufficed to produce an estrous cycle and positive mating.

In experiment V, 14 rats were thus treated, together with 14 controls which also were placed on diet 30 but received Z supplement regularly from the 60th day of age and an additional 12 drops late in pregnancy between the 17th and 20th day. Four of the deficient rats failed to become pregnant and were found to have pulmonary infection. One positive mating was followed by a resorption. Nine litters were born, totaling 69 rats. Two entire litters and members of 5 others were found dead on the day of birth, a total of 22 rats. A careful watch was kept so that all rats except 2 were recovered before maternal ingestion. All living young were killed at once and all 67 of the recovered rats were autopsied (Table V). Twelve rats of 4 litters showed a diaphragmatic hernia. Eleven of the control rats gave birth to litters, totaling 78 young; of these, 4 rats, one from each of 4 litters, had hernias. The malformation could therefore be reproduced in a fair proportion of the young bred on the standard regime, but it occurred also in the control litters.

Experiment VI. In experiment VI further proof that a deficiency of vitamin A increased the incidence of the malformation was sought by several methods. The first of these was the breeding of 5 successive generations of rats on diet 30 with supplement Z, to determine the incidence of hernia and also to test severely the adequacy of the diet. The sixth generation was divided into 2 groups, one of which was placed

on diet 30 + E and the other, composed of littermate controls, on diet 30 + Z, the supplements being started at 60 days of age. Facilities did not permit the maintenance and breeding of all rats of 6 generations. F_1 consisted of 8 females and 4 males picked at random from the stock colony; therefore the experiment was not based strictly on inbreeding. In subsequent generations, brother and sister matings were frequently but not uniformly made; breeding was always with the animals of the

TABLE III

Experiment VI. The Incidence of Diaphragmatic Hernia in the Young of Rats Bred for Five Generations on Diet 30 + Z as Compared with the Sixth Generation, Half of Which Were Bred on Diet 30 with Supplement and Half Without. All but One of the F_6 Experimental Rats Had Ceased to Have Estrous Cycles and a Single Estrus Was Induced by One or More Doses of Two Drops of Z (250 i.u. of Vitamin A).

Generation	Bred on diet	Number of litters		Number of rats	
		Total	With D.H.	Total	With D.H.
F_0	Stock			8	1
F_1	30 + Z	8	2	35	2
F_2	30 + Z	7	1	34	2
F_3	30 + Z	9	2	60	2
F_4	30 + Z	9	3	51	6
F_5	30 + Z	9	1	35	2
F_6C	30 + Z	14	1	108	1
Total	Diet 30 + Z	56	10	323	15
F_6 Exp.	Diet 30	14	5	92	17

same generation for this experiment. In the first 5 generations a number of young were saved for breeding and the remainder killed. All were ultimately autopsied. Of a total of 415 rats, 14 were found to have a diaphragmatic hernia (Table III). In the sixth generation the females of 8 litters were distributed into 2 groups of 15 rats each, dividing the littermates as evenly as possible. There were 12 pairs of littermates. Fourteen rats of each group produced litters. The young were killed on the day of birth to prevent destruction of the evidence. Of the 92 rats in the experimental litters, 17 had hernias of the diaphragm, while one of the 108 rats in the control litters had this malformation (Table III). Therefore, it was apparent that, although the appearance of hernia was suppressed in large part for successive generations on an adequate diet, it reappeared in a generation given a deficient diet. The adequacy of diet 30 + Z supplement is attested by the high fertility and good growth and appearance of the rats maintained on it for 6 generations.

Experiment VII. In experiment VII, wherever possible, the rats

which had produced a number of young with hernias when bred on a deficient diet were given supplement Z three times a week at levels of 7 drops weekly for several weeks, while diet 30 was continued. They were then bred again, if possible to the same males, and the litters examined for hernias. In no instance did these second litters include a rat with a hernia (Table IV). The tendency to herniation was apparently suppressed in the presence of high dietary levels of vitamin A.

TABLE IV

Experiment VII. Comparison of the Incidence of Diaphragmatic Hernia in the Litters of Rats Maintained on a Deficient Diet with Subsequent Litters of the Same Rats after Vitamin Supplements Had Been Given.

First litter, diet 30; no supplement until late in pregnancy						Second litter, diet 30 with Z supplement					
Female	Male	Age	No. of young	No. for post-mortem	No. with D.H.	Z supplement from age	Male	Age	No. of young	No. for post-mortem	No. with D.H.
		days				*days*		*days*			
3654	3598	99	5	5	5	95	3598	136	2	1	0
3658	3597	99	9	9	9	94	3597	135	11	4	0
4413	4174	147	9	9	6	142	4157	188	9	9	0
4424	4174	142	6	6	1	141	4162	199	8	8	0
4429	4132	144	6	6	1	143	4162	184	9	8	0
4442	4117	104	11	11	8	100	4157	190	9	8	0
4297	3946	135	2	2	1	128	3953	238	3	3	0
4307	4112	130	7	7	3	124	4024	231	5	5	0
4333	4226	94	6	6	2	89	4226	158	9	9	0
Total 9 litters			61	61	36	9 litters			65	55	0

Comparison of the incidence of hernias among autopsied rats in first and second litters gives $X^2 = 51.3$, $P = <.01$.

Experiment VIII. In experiment VIII, in order to determine whether the appearance of hernias under these dietary conditions was a characteristic confined to our inbred strain of stock rats, experiment VI-*b* was twice repeated, using inbred rats of the Long-Evans strain. These rats were black, having been selectively inbred in this regard for the purpose of making sure that there was no accidental admixture with the white stock colony. The first batch was run in April and May and the second in September and October, 1941. The data are presented in Table V. One hernia was found in the young of one of the autumn experimental group and none were found in the controls. There is thus a marked difference in these two strains of rats in the tendency for diaphragmatic hernia to appear under the influence of vitamin A deficiency.

Experiment IX. An attempt was made in experiment IX to discover the genetic characteristics of the malformation, by producing a sub-

strain in which the incidence of hernias was uniformly high under experimental conditions. For this purpose female rats nos. 3654 and 3658 were chosen (Table VI). These rats had been part of experiment IV-*b* and had produced 5 and 9 young, respectively, all of which had

TABLE V

Comparison of the Incidence of Diaphragmatic Hernia in the Stock, D.H., and Long-Evans Strains on Diet 30 with and without Z Supplement.

Strain	Experiment	Experiment or control	Rats	Litters	No. autopsied	No. with D.H.	Percentage with D.H.	X^2 and P
Stock	V	Experiment	14	9	67	12		
		Control	14	11	78	4		
	VI F_6	Experiment	15	14	92	17		
		Control	15	14	108	1		
	Total	Experiment	29	23	159	29	18.2	$X^2 = 23.4$
		Control	29	25	186	5	2.7	$P = <.01$
D.H.		Experiment	9	7	34	14		
		Control	8	8	46	5		
	Carotene	Experiment	12	8	55	16		
	experiment	Control	12	11	84	5		
	Total	Experiment	21	15	89	30	33.7	$X^2 = 23.2$
		Control	20	19	130	10	7.7	$P = <.01$
Long-Evans	Spring	Experiment	12	10	68	0		
		Control	13	13	87	0		
	Fall	Experiment	11	8	43	1		
		Control	11	11	66	0		
	Total	Experiment	23	18	111	1	0.9	
		Control	24	24	153	0	0	$P = <.01$

Comparison of the stock and D.H. strains on the experimental regime gives $X^2 = 7.53$, $P = <.01$; and of these strains on the control diet comparison gives $X^2 = 5.5$, $P = <.02$. The infrequency of hernia in the Long-Evans strain is apparent.

had right diaphragmatic hernias, 13 of them complete. These rats had been littermates from the stock colony and had been placed on diet 30 at 36 days of age. They were mated to males nos. 3598 and 3597, respectively, which were littermates. All 4 of these rats were without hernias. After their participation in experiment IV-*b*, the females were continued on diet 30 and given 7 drops of Z weekly and partook in experiment VII, producing small litters in which no hernias were found. They were then both placed on stock diet and after several weeks were each bred twice more, all 4 litters being sired by rat no. 3598. These 3 rats, 3654, 3658, and 3598, are considered as the F_1 generation of the D.H. strain. The resulting strain produced a larger number of members with hernias than the original stock strain, but the incidence still varied with the diet. With the use of this D.H. strain some infor-

mation as to genetic pattern of the malformation was obtained (Text-Fig. 1 and Table VII). On the stock diet in a total of 328 rats, 107 (or 32.6 per cent) were found to have a hernia. The sex distribution was approximately equal, with 50 of 164 males and 57 of 156 females affected; the sex was not recorded in 8 unaffected rats. Of these hernias,

TABLE VI

Breeding Record of Rats 3654 and 3658, of Experiment IV. The D.H. Strain Was Derived from Litters 3 and 4.

Female	Litter	Male	Diet	Supplement	Parturition		No. born	No. for post-mortem	No. with D.H.
					Age	Day of pregnancy			
					days				
3654	1	3598	30	4 Z at 19th day	99	24	5	5	5
	2	3598	30	7 Z per week	136	24	2	1	0
	3	3598	Stock	0	217	24	9	7	1
	4	3598	Stock	0	308	?	4	4	1
3658	1	3597	30	2 Z on 19th day	99	25	9	9	9
	2	3597	30	7 Z per week	135	23	11	4	0
	3	3598	Stock	0	219	23	10	10	3
	4	3598	Stock	0	295	23	9	9	4

34 were pericardial, 13 were right incomplete, and 3 were right complete in the males; 49 were pericardial, 6 were right incomplete, and 2 were right complete in the females, a sex difference which is probably not significant.

The incidence of hernias in the young is significantly greater when both parents are affected than when neither is affected, while the inci-

TABLE VII

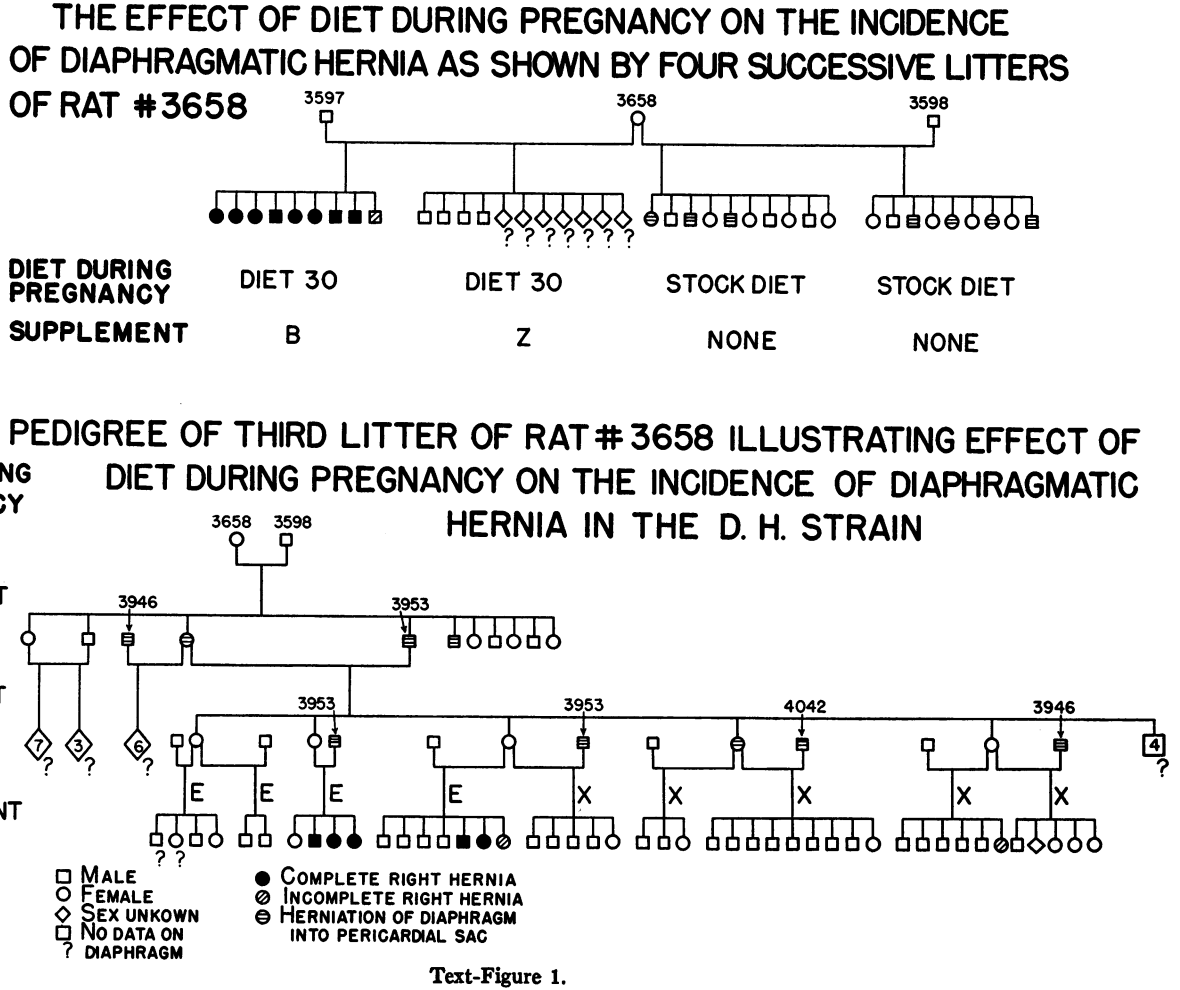
Experiment IX. Incidence of Hernias in the Young of the D.H. Strain in Relation to the Presence of Hernia in the Parents. Stock Diet.

Hernia in parents		No. of litters	Young						Total		Percentage with D.H.
			Normal			Peric. D.H.		Other D.H.			
m.	f.		m.	?	f.	m.	f.	m.	f.	Rats	D.H.	
None	None	19	50	4	37	7	12	3	3	116	25	21.6
Peric.	None	14	16	4	19	8	9	1	5	85	27	31.8
None	Peric.	4	10		9	3	1	0	0			
Peric.	Peric.	16	27		25	8	17	8	0	85	33	38.8
?	None	5	11		9	8	10	4	0	42	22	52.4
Total		58	114	8	99	34	49	16	8	328	107	32.6

Comparison of incidence of hernia in young when both parents are affected in contrast to neither parent affected gives $X^2 = 7.1$, $P = <.01$. Comparison of litters of one versus no affected parent gives $X^2 = 2.6$, $P = <.10$. The former is significant, the latter is not.

Peric. = pericardial hernia.

dence when only one parent is affected is intermediate between these two. The genetic pattern is therefore neither recessive nor dominant



Text-Figure 1.

and is not that of a sex-linked character. The malformation appears to be either the result of a general tendency within the strain or dependent on a number of genes.

Experiment X. The dietary factor which suppresses the expression of the hernia has been assumed to be lack of vitamin A, because no other nutritional substance is known to be present in effective quantities in the small amounts of haliver oil which were used. At the time that these experiments were performed, crystalline vitamin A was not available. Beta carotene therefore was used as a source of vitamin A

TABLE VIII

Experiment X. The Incidence of Diaphragmatic Hernia in the Young of Rats of the D.H. Strain Given Diet 30 with Supplements E, Z, or Carotene.

Supplement	No. of litters	Rats			Type of D.H.			
		No. D.H.	With D.H.	Percentage with D.H.	Peric.	Rt. compl.	Rt. inc.	Left inc.
Carotene	10	63	11	17.5	0	2	8	1
30 + Z	11	79	6	7.6	1	0	5	
30 + E	8	39	16	41.0	0	4	12	

Comparison of the Groups

Supplement	X^2	P
E vs. Z	12.4	<.01
E vs. B carotene	3.9	<.05
Z vs. B carotene	2.5	>.10

in an experiment designed to settle this point (experiment X). Rats of the D.H. strain were used, and were divided into 3 groups. They were subjected to the standard experimental procedure, starting the diet and supplements on the 30th day and mating on the 80th day. The supplements used were E, Z, and beta carotene dissolved in Wesson oil. The carotene solution contained 3 mg. of carotene per cc., and on the basis of 0.6 γ of carotene being taken as the equivalent of 1 i.u. of vitamin A, this solution had a theoretical potency equal to that of the Z supplement. In the ensuing experiment, hernias were found in 41 per cent of the young of the rats receiving E, in 7.6 per cent of those receiving Z, and in 17.4 per cent of those receiving carotene (Table VIII). The difference between the incidence of hernias in the Z and E groups is statistically significant but the difference between the carotene and E groups is less striking.

As the experiment progressed it became apparent that carotene was not as effective as Z in suppressing the appearance of hernias, and it seemed possible that the difference lay in the utilization of the two substances. The livers of the remaining rats were assayed for their content of carotene and vitamin A; although the data are few, they sup-

port the hypothesis that carotene was poorly utilized (Table IX). The results of this experiment support, but do not prove, the hypothesis that the effective agent is vitamin A. It is to be regretted that it was not possible to repeat this experiment with crystalline vitamin A.

TABLE IX

Experiment X. Assays of the Liver for Carotene and Vitamin A. Diet 30 with Supplements E, Z, or Carotene.

Rat no.	Hernia		Supplement	Liver					Young
	m.	f.		Weight	Carotene	Vitamin A	Carotene	Vitamin A	
				gm.	*γ per gm.*	*i.u. per gm.*	*γ per liver*	*i.u. per liver*	
4842	None	None	E	9.8	0.45	9.3	4.4	91	Missing
4906	Peric.	Peric.	E	9.1	0.27	25.8	2.5	234	Missing
4913	?	Peric.	E	6.6	0.40	3.2	2.6	21	No litter
4835	Peric.	None	Carot.	8.6	0.95	26.1	8.1	228	7 normal, 1 rt. compl.
4836	?	None	Carot.	7.5	1.19	22.3	8.9	167	1 normal, 3?
4843	Peric.	Peric.	Carot.	6.8	0.36	37.6	2.6	264	5 normal, 1 left inc.
4881	None	None	Carot.	9.8	0.42	16.1	4.1	158	9 normal
4891	None	None	Carot.	6.0	0.55	25.4	3.3	152	5 normal, 1 rt. compl., 2 rt. inc.
4897	Peric.	Peric.	Carot.	9.3	0.33	21.8	3.0	202	Missing
4903	None	Peric.	Carot.	7.7	0.41	27.4	3.2	211	Missing
4927	None	None	Carot.	9.9	0.39	19.4	3.8	192	12 normal, 2 rt. inc.
4839	Compl.	None	Z	7.8	0.80	1767	5.9	13,783	3 normal
4847	Peric.	Peric.	Z	7.8	0.29	1463	2.2	11,411	8 normal, 1 rt. inc.
4879	?	Peric.	Z	7.1	0.60	1050	4.3	7,459	9 normal
4880	None	None	Z	10.1	0.41	853	4.1	8,614	5 normal, 1 peric., 3 rt. inc.
4892	Peric.	None	Z	7.6	0.50	987	3.8	7,498	6 normal
4904	Peric.	Peric.	Z	8.0	0.54	915	4.3	7,316	4 normal, 1 rt. inc.
4909	None	Peric.	Z	7.6	0.47	1189	3.5	9,033	10 normal
4921	None	None	Z	6.6	0.50	919	3.3	6,062	Missing

Pathologic Anatomy

The Diaphragm. The hernias of the diaphragm were classified with respect to location and type as follows: (a) Right complete, with communication between the right pleural and peritoneal cavities; (b) right incomplete, consisting of a bulge of intact diaphragm into the right pleural cavity; (c) pericardial, consisting of a bulge of the diaphragm into the pericardial cavity; (d) left incomplete. No herniation through any natural opening such as the esophageal hiatus was observed. A complete defect was found only in the right leaf. A more detailed description of the malformations follows.

(a) A right complete hernia consisted of a defect varying from one involving the posterior half of the diaphragm to one in which the right leaf of the diaphragm was lacking (Fig. 1). In some instances a narrow shelf of muscle along the anterior diaphragmatic attachment remained. In all cases part or all of the right lobe of the liver lay in the right pleural cavity, and behind it, also in the pleural cavity, there were varying amounts of small intestine and the hepatic flexure of the colon. The right lung was small and was pushed up to occupy the upper part of the cavity. The right kidney usually was in its normal position. In one instance the right horn of a pregnant uterus was incarcerated in the right pleural cavity and was the cause of death. In another instance the mass of intestines in the right pleural cavity bulged through the mediastinum behind the pericardium but anterior to the esophagus to lie partly to the left of the spine, separated by a thin membrane from the left pleural cavity.

(b) The right incomplete hernia was found always in the posterior half of the diaphragm, and consisted of a roughly hemispheric sac consisting of pleura and peritoneum without interposed muscle and containing a nubbin of liver tissue which had grown to fill it (Fig. 2). The upper surface sometimes was flattened in the larger hernias. The size varied from one measuring 10 by 10 by 5 mm., involving about one-third of the right leaf, to small protuberances about 1 mm. in diameter. The majority were between 3 and 6 mm. in diameter. Occasionally several of these small bulges were present. The location varied but was always in the posterior half of the right leaf.

(c) The pericardial hernias were similar to the smaller right incomplete hernias except that they usually were more flattened and not always round (Fig. 3). They varied from 0.5 to 7 mm. in diameter, were sometimes multiple, and always contained a nubbin of liver.

(d) The left incomplete hernias were found infrequently, lay in the posterior half of the left leaf, and usually contained the upper half of

the stomach and occasionally a loop of gut, seen through the thin membrane.

The rats having a complete hernia died in the first day or two of life with few exceptions. The incomplete hernias were commonly found in rats bred according to the standard procedure which survived to adult life, and this lesion appeared to have no effect on their health or growth. Only a small percentage of the instances of neonatal death could be attributed to a complete hernia, however, and the cause of death usually was not apparent on gross post-mortem examination.

A comparison of the frequency of hernias of the various types in the young of deficient as compared with control mothers shows a greatly increased number of right complete hernias and a somewhat greater number of right incomplete hernias in the experimental animals, but no significant difference in the pericardial and left diaphragmatic hernias. The following figures summarize the incidence of the various types in all experiments of the stock strain, but do not include the rats of the D.H. or Long-Evans strains.

Type	*Experimental rats*		*Control rats*	
	No.	Percentage	No.	Percentage
Right complete hernia	32	50.8	4	16.7
Right incomplete hernia	28	44.5	12	50.0
Pericardial hernia	2	3.2	7	29.2
Left incomplete hernia	1	1.6	1	4.1
Total	63	100.0	24	100.0

Since many of these rats were observed in earlier experiments, it is probable that many of the pericardial hernias were missed, which may account for the lower incidence of this type in the stock rats as compared with the D.H. strain.

The hypothesis which most reasonably explains the morphogenesis of these hernias is a delayed growth of the diaphragm in the deficient animals. The posterior portion of the diaphragm is the last to close. Normally, the completion of the closure of the diaphragm precedes the return of the gut from the extra-embryonic celom of the umbilical cord. If the differential growth rates of the diaphragm and of the gut were altered so that the gut returned from the cord and increased in size prior to the closure of the diaphragm, it is reasonable to suppose that some abdominal organs might pass through the defect and prevent its closure.

Pancreas and Trachea. In the foregoing experiments it was possible to compare the microscopic changes in the trachea and in the pancreas

in rats bred on the deficient diet and examined at the age of 1 to 3 days with those in rats similarly bred but killed at weaning, in survivors continued on deficient diets, and in controls paralleling each group. It was possible to obtain sections of the organs of only a small number of rats from each experiment. These sections were stained with hematoxylin and eosin.

Of the rats bred of deficient mothers and examined at the age of 1 to 3 days, there were 8 from experiment III, 5 from experiment IV-*a*, and 8 from experiment IV-*b*, a total of 21. None showed any abnormality of the pancreas in the microscopic examination, when compared with 5 young from the stock diet controls. The trachea was examined in 4 rats of experiment IV-*a* and 7 of IV-*b*. Two of the latter showed early squamous metaplasia of the tracheal epithelium but the remaining 9 appeared normal. These 2 rats were among the 6 so examined in which a right diaphragmatic hernia was found.

Some of the rats of experiment IV in which the litters were successfully exchanged were examined microscopically, giving an opportunity to observe the effects of lactation by a deficient mother. No abnormalities of the pancreas were found in the 21 rats examined at the age of 21 to 23 days. Of these, 3 were bred and reared by deficient mothers, 4 were bred by deficient mothers and nursed by normal ones, 10 were bred by normal mothers and nursed by deficient ones, while 4 were normal controls. Squamous metaplasia of the tracheal epithelium was found in 5 of 20 rats examined, all 5 of which had been nursed by deficient mothers, but 4 were bred by normal mothers. Tracheitis was found in 9 more, 7 of which were nursed by deficient mothers. None of the rats bred and reared to weaning on a deficient diet were without either infection or metaplasia of the epithelium, while the 7 with normal tracheas were variously distributed among the other groups. Squamous metaplasia of the renal epithelium was found in only one rat, bred and reared on a deficient diet.

In addition to the above rats bred on deficient diets, there was also a large group from experiment III in which the pancreas was examined, at ages of 1 to 4 months. In none of these was there any suggestion of cystic fibrosis of the pancreas, although the acinar cells were small and atrophic in a few emaciated animals, and a few metaplastic cells were found in the pancreatic ductal epithelium in a few deficient animals. These observations were made on 12 animals bred on A supplement, 9 on B, 6 on C, 6 on D, and 8 on E supplement. In a total of 71 rats bred on levels of supplement leading to high neonatal mortality and an increased percentage of hernia (B, C, D, or E supplements), no pancreas showed changes suggestive of fibrocystic disease.

Metastatic Calcification. A large number of rats which had received diet 30 for a period of 10 weeks or more showed metastatic calcification. Of 44 rats which were killed before the age of 90 days and which had received diet 30 with various supplements, only 3 showed calcium deposits in the kidney, whereas 30 of 54 rats killed after the age of 90 days showed calcification in the renal parenchyma. The majority of these were killed at about 4 months of age. Rats on the stock diet rarely showed this change, and as the calcification was found in rats receiving low (B, C, D, and E) as well as high levels of supplement it was attributed to the diet rather than to hypervitaminosis D. It was found in rats bred on the stock diet and transferred to diet 30 before the 6th week as well as those bred on diet 30. In some of the rats showing calcium deposition in the renal cortex there was also calcification of the media of small arteries in the liver and elsewhere, with morphologic features suggestive of early Mönckeberg's sclerosis. There was no evidence of association of calcification with the hernias.

Cause of Death in the Neonatal Period. Except for the relatively small number of rats with a complete right diaphragmatic hernia, there was no gross pathologic change which explained the high neonatal mortality of the young of deficient animals. Microscopic sections of the lungs, liver, kidneys, spleen, and muscle gave no explanation of death. The majority of the deficient animals which survived to weaning and beyond had diffuse chronic bronchitis and interstitial pneumonia, but these changes were not found in the first few days of life. Observation of the living newborn rats led to the conclusion that the animals were too weak to breathe or to suckle, but they responded to painful stimuli. Whether the weakness was due to deficient function of muscle or of the nervous system was not determined.

Discussion

This series of experiments provides one more example of failure to attain the initial objective of an experiment with an incidental discovery of greater interest than the one originally sought. The general concept that congenital malformations may be produced by means of nutritional deficiency during pregnancy has been established previously. A variety of congenital malformations have been reported as the result of vitamin A deficiency by a number of authors working with various species of animals. It has been difficult to understand why the malformations reported by these workers varied so widely, especially in respect to conditions such as hydrocephalus and cyclopia, which are apparent without the aid of special technics. The present experiments suggest that species and strain differences may account for this.

In rats of the stock and D.H. strains fed on the stock diet, hernias of the diaphragm occurred in an appreciable number of cases. The incidence was increased to about 19 per cent in the stock strain bred on the deficient diet and was reduced to 2.7 per cent by means of generous supplements. In rats of the Long-Evans strain none of the control series and only one on the deficient diet was found to have a hernia. The strain differences are of a degree to be statistically significant. Nutritional deficiency therefore enhanced the degree of manifestations of this hereditary defect. The most plausible hypothesis is that in the affected strain the closure of the diaphragm was late in the strain generally, and that additional delay in the deficient animals led to an increased incidence of hernias.

Hernia of the type found in these rats is occasionally observed in the human subject but is less common than hernias through one of the natural openings of the diaphragm. It would be rash to infer that the deductions made from these experiments are directly applicable to the diaphragmatic hernia of man.

Two general concepts have arisen from these experiments which may prove to have application in the study of human neonatal mortality. The first is that a degree of specific nutritional deficiency during pregnancy may exist which is insufficient to produce obvious ill health in the mothers but which leads to death of the majority of the young; the cause of death may not be apparent on careful gross and microscopic pathologic examination. The second is that a tendency to a congenital defect may be carried as a genetic trait which is infrequently expressed under good nutritional conditions, but is frequently expressed when the diet during pregnancy lacks the appropriate specific nutritional factor. If congenital defects occur in this manner in man, it would be difficult to obtain evidence of the relationship of a specific malformation to a specific deficiency. Under general adverse nutritional circumstances in a population, however, one would expect a rise in neonatal mortality and in the incidence of congenital malformations.

Summary and Conclusions

A method has been devised whereby rats may be bred on a regime approximating the maximal deficiency of vitamin A compatible with reproduction. On this regime the majority of the young die during the first 2 days of life, while the mothers show few or no signs of deficiency.

No explanation of this high infant mortality has been obtained by gross examination in most cases, and microscopic examination of the lungs, liver, kidneys, pancreas, and striated muscle also failed to explain

death. No changes suggestive of cystic fibrosis of the pancreas were found.

Rats of an inbred stock albino strain which were bred on a vitamin A-deficient diet showed a high incidence of congenital diaphragmatic hernia, usually involving the posterior half of the right leaf of the diaphragm. Control animals of the same strain bred on the same diet supplemented with a small amount of haliver oil diluted in vegetable oil showed a low incidence of this malformation.

Evidence that nutritional deficiency during pregnancy was responsible for the malformation was provided by breeding rats first on the deficient diet, then feeding them a generous supplement of vitamin A and breeding them a second time. In the 9 rats of this experiment with a high incidence of the malformations in the first litters, no instances of hernia were found in the second litters.

Evidence that the deficient factor, the lack of which led to congenital diaphragmatic hernia, was in fact vitamin A was provided by two experiments: (a) Rats bred for 5 generations on the deficient diet with a supplement of haliver oil gave a low incidence of the defect, whereas the 6th generation bred without supplement gave a high incidence compared with controls of the same generation. (b) Crystalline carotene gave partial protection. The fact that it was partial was explained by the low levels of vitamin A stored in the livers of these rats, suggesting inadequate utilization of the carotene.

The tendency to diaphragmatic hernia is a genetic trait, since the defect was produced in only one rat of 111 of a second strain (Long-Evans), or 0.9 per cent as compared with an incidence of 18.9 per cent in the stock strain on the deficient diet. A substrain (D.H.) was developed from rats of the original stock strain which had a higher incidence of the defect than those of the original stock strain.

It has been demonstrated that the expression of a genetic trait may be enhanced or suppressed by means of diet during pregnancy. Evidence has been provided pointing to vitamin A as the specific nutritional factor responsible in the present experiments.

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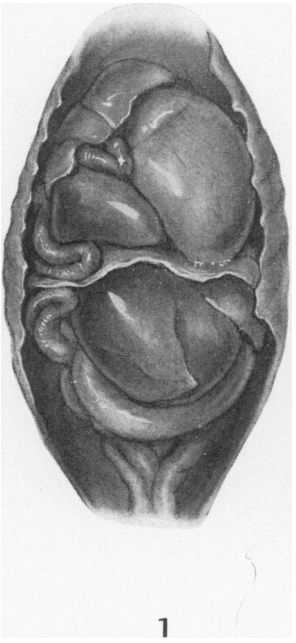
DESCRIPTION OF PLATE

PLATE 23

FIG. 1. Complete right diaphragmatic hernia. The posterior half of the right leaf is absent. Portions of liver and of small intestine lie in the right pleural cavity, compressing the right lung.

FIG. 2. Incomplete right diaphragmatic hernia. The hernial sac is a thin membrane and contains a small nodule of liver.

FIG. 3. Pericardial hernia of the diaphragm. The sac is similar to that of the right incomplete hernia.



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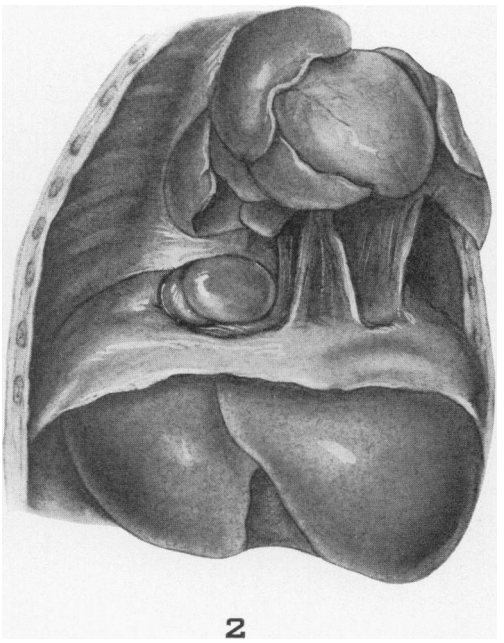

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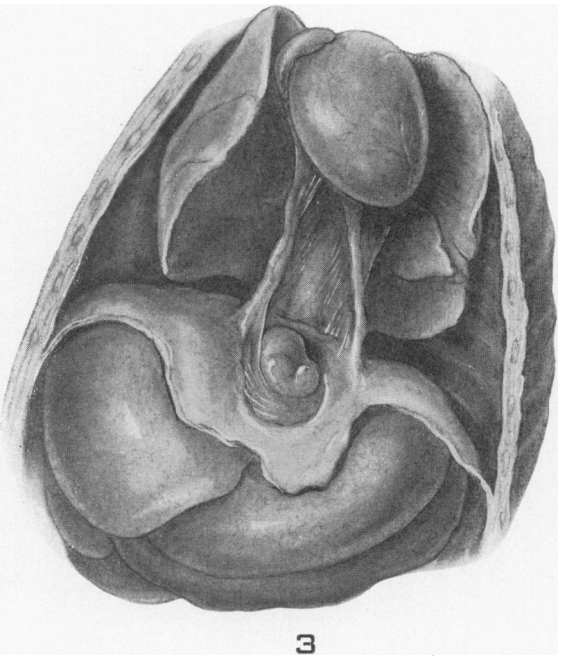

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