LASTING BIOLOGICAL EFFECTS OF EARLY ENVIRONMENTAL INFLUENCES*

V. Viability, Growth, and Longevity

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We have shown in earlier publications that the adult size and metabolic activities of mice can be profoundly and lastingly altered by manipulating the conditions to which these animals are exposed shortly after birth (1–4). The effects of such early influences persist throughout the life span even if attempts are made to correct them by changing the environment after weaning and during adult life. Further illustrations of these phenomena will be described in the present paper.

The new experiments were carried out with animals from a highly inbred mouse colony. This colony was derived from germfree animals which were reassociated with a known microbial flora free of potential pathogens. By controlling the diet of the dam during lactation, treating her at various periods of time with sublethal doses of bacterial endotoxin, shortening or lengthening the period of lactation, or contaminating the young with an enterovirus shortly after birth, it was possible to produce at will highly distinctive progenies. These gave rise to a family of growth curves, the characteristics of which were determined by the conditions to which the animals had been exposed during the preweaning period. The anatomical and physiological characteristics of adult animals could thus be determined by early influences that did not affect either their viability or their longevity.

Materials and Methods

Animals.—Earlier studies in this series were carried out chiefly with specific pathogen-free (SPF) mice of a colony (NCS) developed and maintained in our laboratories (5, 6). In all experiments reported in the present paper, we used instead COBS pathogen-free mice obtained from Charles River Breeding Laboratories, North Wilmington, Mass. As indicated by the initials COBS (Caesarian-obtained, barrier-sustained), these animals are obtained in the germfree state by Caesarian section, then reassociated with a bacterial flora free of pathogens, and maintained in an environment designed to protect them against further

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contamination. In some cases, male and female COBS were mated in our laboratory; in other cases, the females were received during the 1st wk of pregnancy. In all cases, an attempt (usually successful) was made to protect the animals from contamination by housing them in "Isocages" (Lab Cages, Inc., Kennett Square, Pa.), which were covered with an air filter, made of synthetic plastic material. The litters were maintained in these Isocages at least until weaning, and often for longer periods of time.

Diets.—The three different diets that were used were given ad libitum.

D&G pellets: (Dietrich and Gambrill Inc., Frederick, Md.)

Gluten diet: this is the diet described as 20 G in references 1 and 7.

Casein magnesium diet: this is the diet described as 20 C in reference 7; it was supplemented with magnesium chloride to prevent mortality among the pregnant females (1).

General Remarks.—It has been observed empirically that the growth rate of young mice in our experiments was affected by ill-defined environmental factors (weather? light? noise? etc.), even though the animal rooms were air-conditioned (imperfectly) and not used for usual laboratory operations. In order to minimize the influence of these unknown environmental factors on the interpretation of the results, we made it a practice to use only, whenever possible, animals born within a period of 2 or 3 days for each particular test. This required, naturally, the availability of large numbers of pregnant animals, from which we selected the litters born on approximately the same day.

It is well known that the growth rate of the young is also markedly affected by the number of young in the litter. For this reason, we discarded all litters with fewer than six young. This made it necessary to discard the progeny of large numbers of females on nutritionally deficient diets, since these females commonly gave birth to small litters or ate some of their young shortly after birth.

For the sake of uniformity, the numbers of young per litter were reduced to six or eight on the 2nd day after birth. Males were separated from females during the 5th wk of life, and transferred then to other cages; however, all the males (or females) from the same litter were housed in the same cage. The animals were weighed individually, always in the morning. As stated in an earlier publication (1), there were marked differences in weight from litter to litter, but great uniformity within each litter. Males were naturally larger than females, but the trends in weight curves were identical for both groups. For this reason, it appeared justified to limit the presentation of data to the male groups.

Practically all animals that survived the first 3 days were still alive 1 yr later, as indicated in the text figures. This made it possible to establish that none of the early influences introduced to manipulate the growth curves decreased viability or longevity.

The effects of early influences on the growth curves were so profound that there was only very little overlapping between the various experimental groups. This is apparent from the text figures.

RESULTS

A. The Mother's Diet.—We have reported elsewhere that the diet of the mother during gestation and lactation affects the adult size of her young (2). This finding is confirmed in the following experiments carried out with diets and a colony of mice slightly different from those used in earlier studies.

Approximately 1 wk before mating, 30 pairs of animals were placed on either one of the following diets: D&G pellets, casein magnesium diet, or gluten diet.

From each group, four litters born on the same day were selected for comparative study. Each litter was reduced to six young per lactating female on the 2nd day after birth.

The lactating females were maintained on their original diets throughout the period of

lactation; the young continued to nurse until they were weaned from their mother on the 21st day of their life. As usual, however, they began to nibble the solid food available in the cage around the 14th day.

Immediately after weaning, all the young were given D&G pellets, and they remained on this diet continuously throughout the rest of their life span. Fig. 1 shows the weight curves for the males on the three different diets.

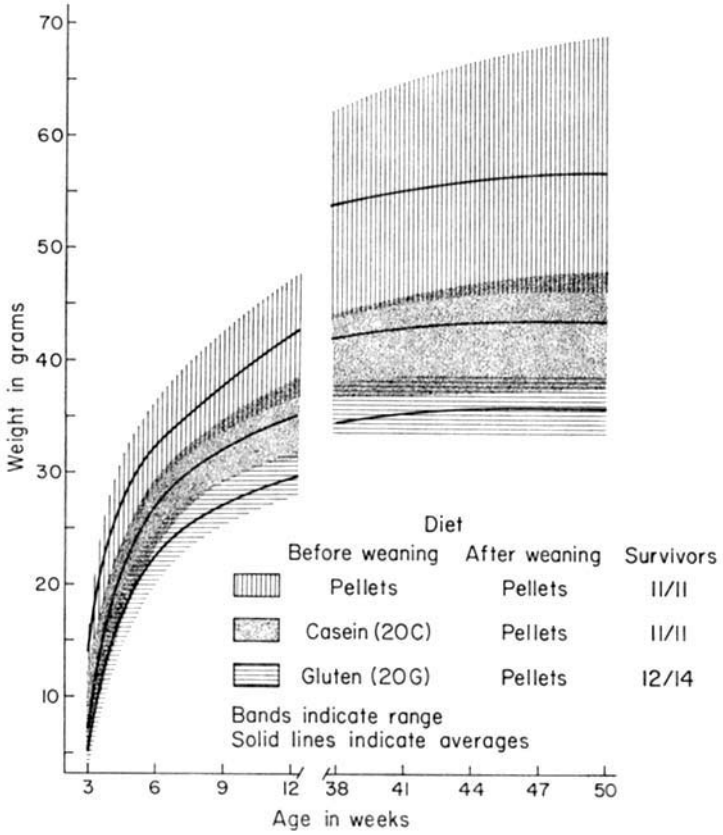

FIG. 1. Effect of mother's nutritional regimen during pregnancy and lactation on adult weight curves and viability of progeny.

The average weights of the males at weaning time were 5.4, 7.9, or 13.6 g for the animals, the mothers of which had been fed, respectively, the gluten, casein, or D&G pellet diets during gestation and lactation. As seen in Fig. 1, the differences in weight persisted from then on, and indeed became larger with time, even though all animals were consistently fed the same diet (D&G pellets) and housed under exactly the same conditions.

The smaller body size of the young resulting from nutritional deficiency

during gestation and lactation did not reduce their viability. Irrespective of size, practically all animals were still living 1 yr after birth, when the experiment had to be discontinued for lack of space. At that time, most of the young born of mothers fed the D&G diet were extremely obese, whereas obesity was not observed in any of the gluten group. Extrapolating from the results of earlier experiments (2), it is probable that most of the obese animals would have died around 18 months of age.

B. The Mothers' Diet During Lactation.—In the preceding experiment, the experimental diets were given to the mothers during gestation and lactation.

TABLE I

Comparative Effects of Diet During Pregnancy and Lactation on the Growth Curve

Diet			Weights* at indicated ages					
During		Post weaning	5 wk		9 wk		13 wk	
Pregnancy	Lactation		Males	Females	Males	Females	Males	Females
			g	g	g	g	g	g
Pellets	Pellets	Pellets	27.4	23.3	35.5	29.3	39.4	32.5
"	Gluten	Gluten	17.3	17.1	28.3	26.7	33.5	30.3
"	"	Pellets	19.2	18.4	31.1	27.3	35.3	31.1
Gluten	Pellets	"	21.3	19.5	29.3	23.5	32.1	35.9
"	Gluten	Gluten	7.5	7.2	14.3	12.7	19.7	18.0
"	"	Pellets	15.3	14.2	28.1	25.9	30.3	26.5

* Averages for approximately 15 animals in each group.

New tests now being carried out indicate that depression of growth of the young can be achieved by limiting dietary restriction to the period of lactation.

This has been repeatedly shown by changing the diet of the lactating female even as late as the 4th day after birth. It will suffice to illustrate the phenomenon here by describing the results of an experiment still under way in our laboratory.

Mating pairs were fed either D&G pellets, or the gluten diet, 1 wk before mating and until birth of the young. The day after delivery, half of the litters were changed from pellets to the gluten diet, or from the gluten diet to pellets, as indicated in Table I. At that time, also, the number of young was reduced to eight per litter. Table I gives the average of individual weights for males and females at 5, 9, and 13 wk of age.

As can be seen in Table I, changing the lactating females from pellets to the gluten diet after birth and throughout lactation depressed the weight curves of their young, even when these were transferred back to pellets after weaning. Changing the lactating females from the gluten diet to pellets immediately after birth corrected in part the depression of the weight curve observed when the gluten diet was continued throughout lactation. These findings demon-

strate that the dietary regimens during both the gestation and lactation periods contribute to the size of the adult.

It is worth noting, also, that when the gluten diet was given after birth, the young of mothers fed pellets during pregnancy grew much faster than those nursed by mothers fed the gluten diet during that period.

C. Duration of the Lactation Period.—Although mice were weaned at 21 days of age in earlier experiments, they started to eat the solid diet available in their cage at a much earlier date. It was noticed indeed that they would all survive even if separated from their mother as early as 13–15 days of age. The results of the following experiment show, however, that shortening of the lactation period usually resulted in a smaller adult size.

In this experiment, all animals were fed D&G pellets throughout the period of pregnancy, but some of them were changed to the gluten diet the day their young were born. Each litter was reduced to eight young on the following day. Weaning took place either on the 15th or 21st day after birth for the pellet group and on the 21st or 24th day for the gluten group. After weaning, all animals were fed D&G pellets exclusively for the rest of their life span. Fig. 2 shows the average weight curves for the males of each of the four subgroups.

In agreement with what had been observed in earlier experiments, the animals nursed by females fed the gluten diet during the lactation period were much smaller at 3 wk of age than were animals nursed by females fed pellets continuously. The average respective weights were approximately 6 g vs. 13.5 g. The new finding was that, in both diet groups, the animals left with their mothers for the longer period of time were not only larger at weaning time, but also became larger adults (Fig. 2). This is probably due to the fact that, even though all young animals began to nibble at solid food around the 14th day of their lives, they also continued to nurse as long as they were left in association with their mothers.

D. Oral Infection of Newborn SPF Mice with an Enterovirus.—We have reported earlier that the growth of SPF mice can be lastingly depressed by infecting them orally within a few days after birth with a filterable agent separated from the intestinal tract of ordinary mice obtained from commercial farms (2–4). For the sake of convenience, this agent has been designated as "enterovirus", although it has not yet been identified (4). The following experiments present further information concerning the effect of the viral infection on the weight curves of animals and on their viability.

Large numbers of SPF mice (1–2 days old) were infected orally several months ago with material derived from tissue cultures of the enterovirus (2, 3). The intestines of these animals were collected aseptically 6 days after oral infection, then immediately frozen at −70°C. Such frozen material has been used as infective material in the three experiments, the results of which are illustrated in Figs. 3 and 4, and in Table III.

The frozen intestines (from young infected animals) were rapidly homogenized in a Teflon grinder, using 5 ml of Tris buffer per intestine. The homogenates (pooled for each particular

experiment) were filtered immediately through a Millipore membrane of 0.45 μ porosity, and the filtrate used the very same day for infection experiments. It is essential to use the filtrate as soon as possible after its preparation because it loses most of its infective activity when kept in the refrigerator.

Two drops of undiluted filtrate were administered orally to COBS mice 1 day after their birth. Each litter was reduced to 8 young on the following day. The diet consisted of D&G pellets and water, given ad libitum. The infected animals were weighed individually at weekly intervals. Figs. 3 and 4 show the trends in weight curves for the males of control and in-

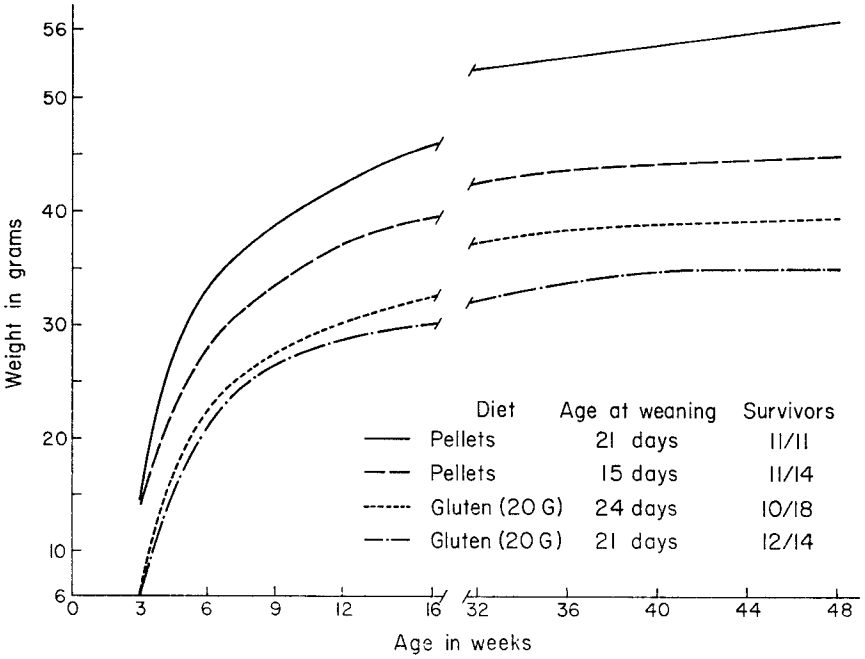

FIG. 2. Effect of length of lactation on adult weight curves and viability, with two different diets.

fected groups in two different experiments. They also show the numbers of survivors 50 wk and 18 months after the beginning of the experiment.

As found in earlier experiments, neonatal infection with the enterovirus resulted in a marked and lasting weight depression. The average difference between control and infected groups was consistently 3–4 g per animal at weaning time, and it became larger as the animals became older. (See also Table III). The viral infection, however, did not kill any animal, even during its early most acute phase. As indicated in Fig. 3, practically all animals were still living 50 wk after infection. Furthermore, the percentage of survivors was exactly the same, 60%, in the control and infected groups, when the experiment illustrated in Fig. 4 was discontinued more than 18 months after infection.

E. Bacterial Infection of Newborn Mice.—It is easy to establish extensive infection or newborn SPF mice with a variety of bacterial species. In contrast with what has been observed after neonatal infection with the enterovirus, however, none of the bacterial species that have been tested caused a lasting weight depression.

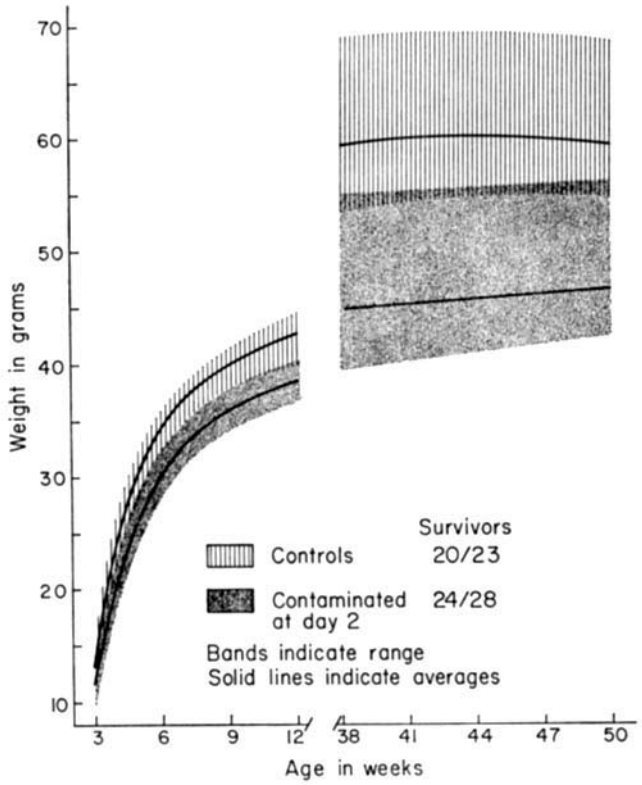

FIG. 3. Effect of neonatal infection with enterovirus on adult weight curves and viability (50 wk).

In numerous experiments that will not be described in detail here, newborn mice from two SPF colonies (NCS and COBS) were infected orally with various strains of enteropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. A very large percentage of infected animals died when the infective dose contained more than 10^7 living bacilli. By using smaller infective doses, in contrast, it was possible to establish consistently a massive and generalized infection of the whole gastrointestinal tract, without causing any mortality. This could be done by the oral administration of 10^3–10^6 bacilli. Very large numbers of bacilli could then be recovered from various organs during the first 2 wk after infec-

tion. By weaning time, however, most bacilli had disappeared except for a few persisters (8, 9).

The growth of animals infected neonatally with enteropathogenic *E. coli* and *K. pneumoniae* was somewhat depressed during the acute phase of the infection; but this effect was transient. At the time of weaning and from then on, no difference in weight could be detected between the control groups and the mice that had been contaminated shortly after birth.

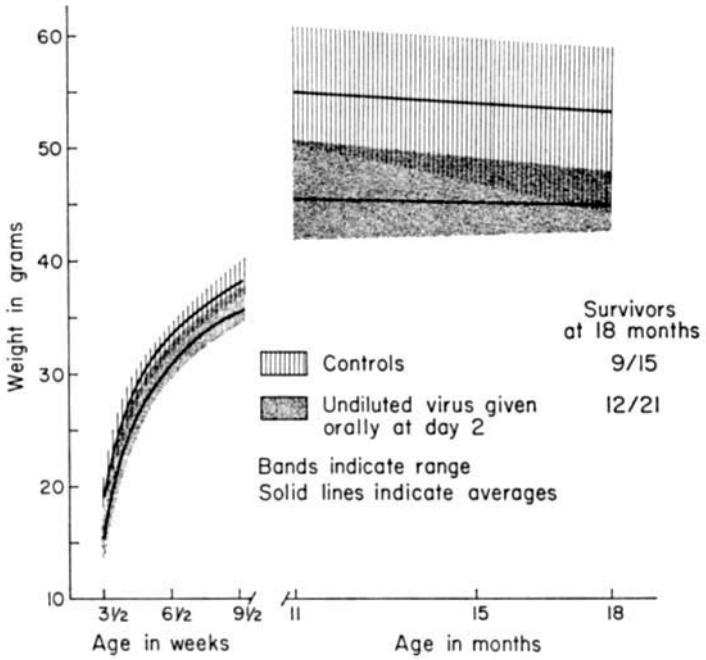

FIG. 4. Effect of neonatal infection with enterovirus on adult weight curves and viability (18 months).

Extensive experiments have also been conducted in this laboratory with mycobacterial infections of newborn mice. Small doses of virulent tubercle bacilli, and large and small doses of a highly active strain of BCG, have been administered by the intravenous, the intraperitoneal, or the aerosol routes. These experiments will be described in detail elsewhere.[1] Suffice it to state here that neonatal infection with virulent or attenuated (BCG) mycobacteria consistently resulted in an extensive bacterial invasion of the various organs as well as in immunological and pathological manifestations. Yet, the weight curves of the animals have not been affected by these early mycobacterial infections.[1]

[1] Costello, R., T. Izumi, and T. Sakurami. Private communication.

F. Administration of Bacterial Endotoxin to the Mother During Pregnancy or Lactation.—The failure to affect growth rates by neonatal administration of enterobacilli or mycobacteria does not rule out the possibility that bacterial infections of the mother can have a growth-depressing effect on her progeny. This possibility is indeed substantiated by the effects resulting from administration of bacterial endotoxin to pregnant or lactating mice.

Intravenous administration of endotoxin at any time during pregnancy caused abortion in a large percentage of cases. Because of the difficulties experienced in standardizing either the dose or the time of administration of endotoxin, so as to prevent abortion, it has not yet been possible to design experiments defining in a reproducible manner the effects of endotoxin on the growth of the fetus and the newborn. This aspect of the problem will therefore not be considered further in the present paper.

In contrast, reproducible, striking, and lasting depression of growth of the young has been readily achieved by administering bacterial endotoxin to the mother during the period of lactation. This effect can be achieved without mortality among either mothers or their young. It is worth recalling in this regard that COBS mice, like NCS mice, are highly resistant to the lethal effect of endotoxin.[1]

The endotoxin used was derived from *Escherichia coli* (lot 439277 obtained from Difco Laboratories, Inc., Detroit, Mich.). It was dissolved in physiological saline and the selected dose was administered intravenously (caudal vein) in a volume of 0.2 ml. The experiment was carried out with a single dose of 125 μg endotoxin, administered to the lactating mothers 2, 9, or 15 days after birth of their young. The young were weaned at 21 days of age, weighed individually at that time and at regular times thereafter. The diet consisted of D&G pellets and water, given ad libitum throughout the experiment.

As seen in Fig. 5, administration of a single dose of endotoxin resulted in marked and lasting depression of body weight of the young. In this and other similar experiments, maximum weight depression was achieved when the endotoxin was administered to the mother on the 8th–12th day of lactation.

It is known that endotoxin causes marked vascular constriction, an effect which probably interferes with milk secretion and thereby indirectly with the nutrition of the young. With the dose of endotoxin used, on the other hand, this effect lasts only for some 48 hr. This may explain why the most profound effects on the weight curve were observed when the endotoxin was administered on the 8th–12th day of lactation, a time when the nutritional needs of the young are very high and when the latter are not yet able to eat solid food.

It will be seen in Fig. 5 that, as in the case of nutritional deficiency of the mother or neonatal infection of the young with the enterovirus, a marked and lasting depression of weight by bacterial endotoxin could be achieved without causing any mortality.

G. Experimental Manipulation of the Growth Curve.—The manipulation of the various early influences described in this and other publications makes it

possible to elicit at will a family of markedly different growth curves, with animals derived from a highly inbred mouse colony and therefore having marked genetic uniformity. It will suffice to document this statement with the results presented in Fig. 6, which illustrates that both the size at the time of weaning and the adult size can be made to differ strikingly by manipulating the dietary regimen.

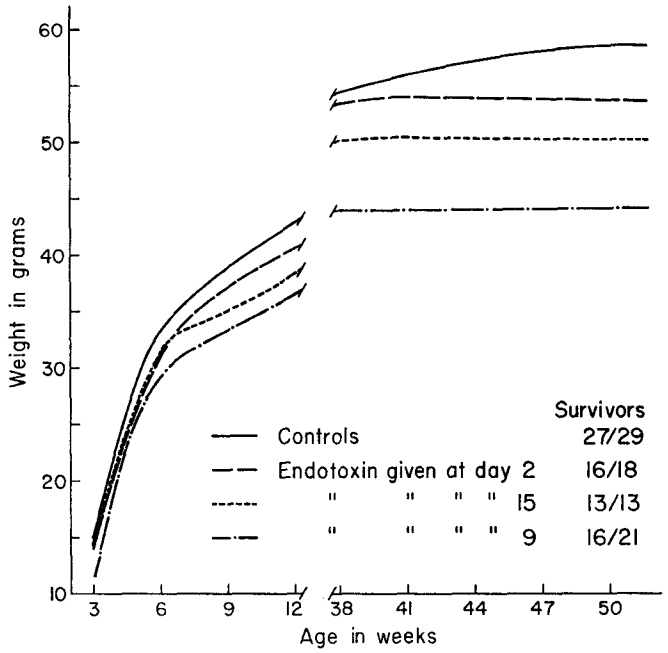

FIG. 5. Effect of endotoxin administered to mother during lactation on weight curves and viability of progeny.

In this experiment, the lactating mothers were fed either D&G pellets or the gluten diet. After separation from their mothers, some of the progeny in the preweaning pellet group were shifted to the gluten diet, and some in the preweaning gluten group were shifted to pellets. The weight curves shown in Fig. 6 refer to the males of each subgroup.

As seen in Fig. 6, large differences could be consistently produced in weaning and adult size by nutritional manipulations. Depending upon qualitative differences in the diet during and after lactation, the weights of the adults could be made to be less than 30 g or more than 50 g. Similar and even greater differences could be elicited by introducing into the experimental system other types of early influences such as: neonatal viral infection, hormonal manipulations, toxic substances, or behavioral disturbances. General reviews of some of these effects will be found in references 11 and 12.

Let it be emphasized again that profound, predictable, and lasting alterations of growth rates can be achieved without causing mortality or affecting the longevity of the experimental animals.

H. Effect of Early Influences on the Comparative Development of Various Organs and Metabolic Activities.—Measurement of total body weight constitutes a convenient method for evaluating the effects of various environmental

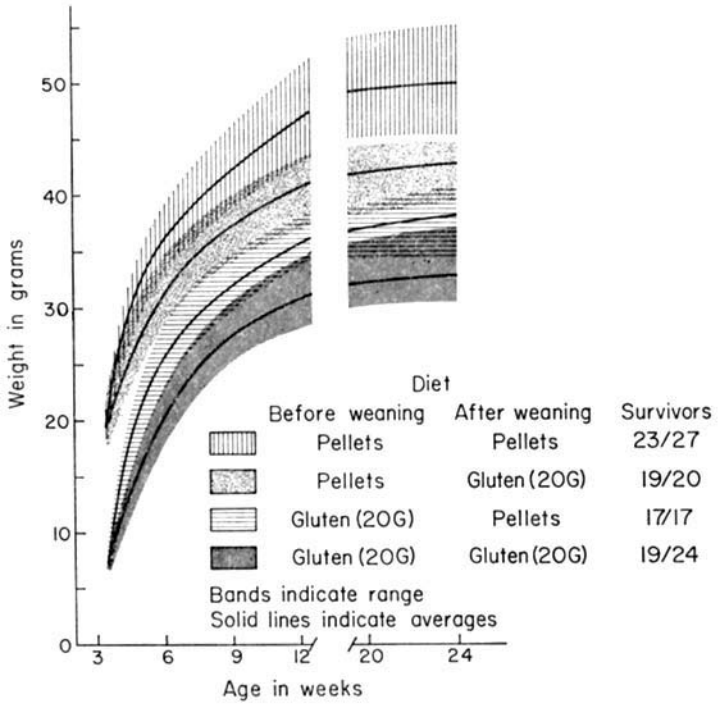

FIG. 6. Effect of mother's diet during lactation and of progeny's diet after lactation on weaning weights and adult weight curves of progeny.

influences on development. But the study of other indices would certainly reveal more fundamental manifestations of these influences. As was shown in an earlier publication, for example, neonatal infection of the mouse with the proper enterovirus results in profound metabolic alterations which persist in adulthood (3–5). More recent studies have revealed that manipulations of the mother's nutritional regimen during lactation can also have lasting effects on the metabolic characteristics of her progeny.[2]

The weights of the individual mouse organs are not uniformly affected by

[2] Lee, C.-J. Data to be published.

the early influences that condition adult size. Tables II and III illustrate the effects of nutritional manipulations and of neonatal infection with the enterovirus on the weight of the whole animal, and of its various organs.

TABLE II

Effect of Mother's Diet During Lactation on Comparative Growth of Various Organs in Her Progeny

Diet			Total weight*			Percentage of body weight		
			at following times after weaning					
Pre-weaning	Post-weaning		4 days	4 wk	10 wk	4 days	4 wk	10 wk
			g	g	g			
Pellets	Pellets	Body weight	25.0	30.6	33.4	—	—	—
		Brain	0.50	0.52	0.52	2.08	1.72	1.58
		Heart	0.16	0.20	?	0.65	0.67	?
		Lungs	0.19	0.22	?	0.77	0.79	?
		Spleen	?	0.10	0.11	?	0.35	0.33
		Liver	1.75	1.90	1.86	7.02	6.22	5.56
Gluten	Pellets	Body weight	12.5	22.3	23.9	—	—	—
		Brain	0.43	0.43	0.42	3.42	1.89	1.70
		Heart	0.09	0.14	?	0.75	0.64	?
		Lungs	0.12	0.16	0.16	1.08	0.72	0.69
		Spleen	?	0.07	0.06	?	0.36	0.27
		Liver	0.86	1.39	1.22	6.80	6.31	5.11
Pellets	Gluten	Body weight	21.4	27.5	31.5	—	—	—
		Brain	0.50	?	0.50	2.36	?	1.59
		Heart	0.15	0.17	?	0.73	0.76	?
		Lungs	0.18	0.20	0.23	0.87	0.82	0.75
		Spleen	?	0.10	0.10	?	0.36	0.33
		Liver	1.32	1.38	1.55	6.14	5.46	4.93
Gluten	Gluten	Body weight	10.9	17.9	23.2	—	—	—
		Brain	0.41	?	0.45	3.88	?	2.05
		Heart	0.08	0.11	?	0.76	0.69	?
		Lungs	0.10	0.15	0.14	0.96	0.89	0.61
		Spleen	?	0.07	0.06	?	0.37	0.28
		Liver	0.56	1.03	1.00	5.12	5.48	3.62

* Figures correspond to averages for 10–20 animals in each group. The organs were weighed in the wet state, within 1 hr after being separated from the animal.

Special emphasis must be placed here on the comparative size of the brain in the different experimental systems. It will be noted that the brain is proportionately much heavier in relation to total body weight, in animals exposed to early influences causing depression of growth, than in control animals. From this point of view, the brain is better protected than other organs against certain types of environmental insults. But the *absolute* weight of the brain was

abnormally small, nevertheless, in all animals that had been exposed to unfavorable conditions during the early phases of their development. As seen in Table II, for example, the brains of adult animals that had been nursed by mothers fed the gluten diet weighed only 0.41–0.45 g, even when these animals had been fed pellets after weaning, while the brains of adult animals that had been nursed by mothers fed pellets weighed 0.50-0.52 g, even when these animals had failed to grow at a maximum rate because they had been fed the gluten diet after weaning. The results shown in Table III similarly show that the absolute weight of the brain is reduced throughout the whole life span by

TABLE III

*Effect of Early Nutrition and Neonatal Infection on Comparative Growth of Various Organs**

	Diet during pregnancy and lactation			Neonatal infection‡
	Pellets	Gluten	50% Restricted	
	Organ weights(*g per 100 g body weight*)			
Brain	1.57 ± 0.031§	1.84 ± 0.014‖	2.05 ± 0.044‖	1.85 ± 0.029‖
Heart	0.46 ± 0.012	0.43 ± 0.025	0.45 ± 0.012	0.44 ± 0.013
Lungs	0.70 ± 0.031	0.68 ± 0.020	0.70 ± 0.012	0.68 ± 0.013
Spleen	0.29 ± 0.013	0.34 ± 0.025	0.38 ± 0.013‖	0.42 ± 0.029‖
Liver	7.10 ± 0.151	7.12 ± 0.301	6.83 ± 0.194	7.31 ± 0.173
Kidneys	1.59 ± 0.024	1.32 ± 0.063‖	1.39 ± 0.077‖	1.64 ± 0.026
	Total body weights			
	32.9 ± 0.77	23.6 ± 0.55‖	23.5 ± 0.32‖	26.0 ± 0.31‖

* Average for 7 males in each group, 6 wk of age.

‡ All infected animals were fed pellets. Details of the techniques used for the neonatal infection are given in section D of the text.

§ Mean of respective group ± standard error of mean.

‖ $P < 0.01$

undernutrition of the mother during the period of lactation, and by neonatal contamination of the young with the enterovirus. Chemical studies, to be reported later, demonstrate that the quantitative differences in brain size are accompanied by marked qualitative differences in the composition of various brain proteins.

SUMMARY

The effects of neonatal influences on the growth and longevity of mice were studied by using animals derived from a highly inbred germfree colony that had been reassociated with a microbial flora free of known pathogens.

The size of the animals at weaning time could be conditioned predictably

by manipulating the diet of their mothers during gestation and lactation or by shortening or lengthening the period of lactation.

A deficient diet during gestation or during lactation decreased the metabolic efficiency of the adult animal, even if it was fed an optimum diet after weaning. The effect was greatest when malnutrition occurred during both pregnancy and lactation. In contrast, an optimum diet during gestation and lactation rendered the animal less susceptible to the depressing effects of nutritional deficiency during adult life.

A marked and lasting growth depression could be reproducibly achieved by contaminating newborn mice orally with an unidentified enterovirus. But neonatal infection with enterobacteria or mycobacteria even though severe, did not significantly alter the growth rate.

Regardless of its initial cause, the depression of the growth rate during the preweaning period persisted throughout the whole life span of the animals, even when they were placed under optimum sanitary and nutritional conditions after weaning.

Agencies (nutritional or infectious) which brought about a depression of whole body weight also affected the absolute and relative sizes of the various organs, *especially of the brain.*

By manipulating neonatal influences, it was possible to produce at will in a given colony of highly inbred mice a family of strikingly different growth curves. This could be done without causing the death of any animal or affecting longevity.

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