# LASTING BIOLOGICAL EFFECTS OF EARLY ENVIRONMENTAL INFLUENCES*

## VI. Effects of Early Environmental Stresses on Metabolic Activity and Organ Weights

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As reported in earlier papers, we have studied separately the effects of various forms of malnutrition during pregnancy and lactation, or of neonatal infection with enteroviruses or enterobacteria, on the growth, viability, and metabolic activities of specific pathogen-free mice (SPF)[1] (1–4). In the present paper we are reporting the effects of early malnutrition combined with neonatal enterovirus infection on the weight gain and on the metabolic turnover of $^{14}$C-acetate and $^{14}$C-glucose of various organs.

### *Materials and Methods*

*Animals.*—All experiments were carried out with SPF mice of the COBS strain obtained from Charles River Breeding Laboratories, North Wilmington, Mass. The animals were maintained in "Isocages" as described in reference 4.

*Infection.*—The enterovirus preparation used in this study is described in references 2 and 5. The infective material was prepared from the intestines of 1-wk old mice which had been orally contaminated with the virus 2 days after birth. The intestines were homogenized with a Teflon grinder in tris hydroxy methylamino methane (Tris)-buffered salt solution; the homogenate was passed through a Millipore filter of 0.45 $\mu$ porosity. In all experimental infection tests, the filtrate was administered orally to 2-day old COBS mice in a dose of approximately 0.05 ml of 100-fold dilution of the virus.

*Diets.*—The composition of the gluten diet is given in reference 1. As found in earlier experiments, the dams raised on this gluten diet usually destroy many if not all of their young. This accident can be prevented by supplementing the gluten diet with small amounts of lysine and threonine. In the present experiment, 0.01% lysine and 0.002% threonine was added to the standard gluten diet.

The dams were placed on the gluten-lysine-threonine diet a week before mating and were kept on it throughout pregnancy and lactation. After weaning all animals were transferred to D & G pellets (Dietrich and Gambrill, Frederick, Md.) and kept on this complete diet, ad lib., until termination of the experiment. Control animals were kept on D & G diet throughout the duration of the experiment.

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[1] *Abbreviation used in this paper:* SPF, specific pathogen-free.

*Weights.*—Body weights were determined at weekly intervals always early in the morning. Organs were weighed in the fresh state, after extra fatty tissue had been removed as completely as possible. The tibia were heated in boiling water for 20 min, freed of tissue, and weighed after desiccation.

*Administration of $^{14}$C-Acetate and $^{14}$C-Glucose and Measurement of Radioactivity in Respiratory Carbon Dioxide.*—Experimental and control mice were fasted for 17 hr. Doses of 33 $\mu$Ci/100 g body weight of 0.1 M sodium acetate-1-$^{14}$C (New England Nuclear, Boston, Mass., specific activity 60.8 mCi/mmole) or 33 $\mu$Ci/100 g body weight of 0.05 M D-glucose-UL-$^{14}$C (New England Nuclear, specific activity 180 mCi/mmole), each in a volume of about 0.2 ml, were injected intraperitoneally. Immediately after injection, the animals were placed in 400 ml volume glass jars, which were tightly closed with rubber stoppers and connected with air inlet and outlet. The air flow was passed through a soda lime tube to remove carbon dioxide contamination and was maintained at a flow rate of about 1 liter/min. The respiratory carbon dioxide was collected at various intervals of time, 0–½, ½–1, 1–2, 2–3, and 3–5 hr after injection into 1 N sodium hydroxide, 5 ml per tube.

In order to determine the quantity of carbon dioxide trapped as sodium carbonate, various amounts of the sodium hydroxide solution used in the traps and samples of each of the five collecting tubes at various times were titrated with 0.1 N hydrochloric acid, using bromocresol green as indicator. The millimoles of carbon dioxide taken up in each tube were calculated from the difference of 0.1 N hydrochloric acid used for the titration of sodium hydroxide solution before and after the uptake of carbon dioxide. Portions of sample solutions were mixed with a slight amount of Hyamine hydroxide (Packard Instrument Company, Inc., Downers Grove, Ill.) and 5 ml of scintillation fluid (6); the radioactivity was counted with a Packard scintillation spectrometer, model 3003.

*Incorporation of $^{14}$C-Acetate or $^{14}$C-Glucose into Total Lipids.*—The animals were killed 5 hr after infection. The liver and brain were rapidly removed, weighed, and homogenized separately in ice-cold distilled water with a Teflon grinder. The total lipids in the tissue homogenate were extracted with two volumes of methanol, twice. The solid residues were added to three volumes of chloroform:methanol (2:1, v/v) mixture, mixed well, and centrifuged. The extraction was repeated twice. The total extracts were combined in a 250 ml beaker and an equal volume of chloroform was added and mixed. The mixture was placed in a 1 liter beaker which was carefully filled with distilled water. The larger beaker was covered with aluminum foil and the preparation allowed to stand overnight. During this time the water-soluble impurities contaminating the lipid extract, as well as methanol diffused into the aqueous layer. After standing overnight, the aqueous layers from both beakers were removed by suction. Most lipids were contained in the chloroform layer in the smaller beaker; a white fluffy layer above the chloroform consisted of certain complex lipids, e.g. sphingolipids. A small quantity of methanol was added to the lipid extract to dissolve the fluffy parts. The purified lipids were condensed to a constant volume. Total lipid content was measured by gravimetry. Portions of samples were added with a slight amount of Hyamine hydroxide to dissolve the lipids and 5 ml of scintillation fluid. Radioactivity was counted with a Packard scintillation spectrometer, model 3003.

## RESULTS

It must be emphasized at the outset that neither early malnutrition, nor neonatal infection, nor the combination of these two stresses, caused any death among the animals used in this study. All litters had been reduced to eight young the day after birth and these eight animals survived until killed for the various experimental procedures. However, as seen in Table I, and in confirma-

tion of earlier results, neonatal infection with the enterovirus depressed the body weights of the animals, as well as the weights of livers and brains (see Figs. 1 *a* and 1 *b* for body weights). A similar result was obtained by feeding the gluten-lysine-threonine diet to the dam during pregnancy and lactation. Depression of weight was still more severe in animals subjected to both early malnutrition and neonatal infection.

The weights of the various organs were not uniformly affected by the early experimental stresses which caused lasting body weight depression. The actual wet weights of liver and brain were smaller in all the experimental groups than in the control group. But relative to the body weight, there was no significant difference in liver weights between infected and control groups. In contrast,

TABLE I

*Effect of Neonatal Infection, Early Malnutrition, and Neonatal Infection Combined with Early Malnutrition on Body and Organ Weights of Male Mice**

| | Controls | Neonatal infection | Early malnutrition | Infection and malnutrition |
|---|---|---|---|---|
| Total Body Weight (g) | 42.1 ± 0.84 | 35.8 ± 0.75‡ | 27.5 ± 0.54‡ | 23.2 ± 0.81‡ |
| Organ Weight (g/100 g body wt) | | | | |
| Liver | 5.03 ± 0.08 | 4.89 ± 0.13 | 4.26 ± 0.13‡ | 4.11 ± 0.19‡ |
| Brain | 1.24 ± 0.01 | 1.41 ± 0.03‡ | 1.81 ± 0.03‡ | 1.86 ± 0.07‡ |

* Averages for 12 males per group, 2½ months old. Mean of respective group ± standard error of mean.

‡ $P < 0.01$.

the liver weights were lower in the progeny from mothers fed the gluten diet, with or without neonatal infection.

Brains were significantly heavier in relation to total body weight in animals which experienced neonatal infection, malnutrition, or both infection and malnutrition combined, than in the control group. Thus, as observed in an earlier study (4), the brain exhibited greater resistance than other organs to early environmental stresses.

A more extensive illustration of the differential effect of early experimental stresses on the various organs is presented in Table II. The figures in these tables refer to the female animals of the various groups, all of them sacrificed at 3–5 months old (when their weights were stabilized), whereas Table I refers to the males 2½ months old.

Table II gives the gross changes in organ characteristics resulting from the various early experimental stresses experienced by the animals. It is apparent once more that organs differ in their response to early stresses and that the brain is least affected as judged from its weight relative to total body weight.

Clearly, also, the worst effects in all organs are seen in animals bred and nursed by mothers fed an inadequate diet and then infected neonatally with the virus. In this case, for example, the liver weighed hardly more than 50% of what it did in control animals and even the weight of the brain was depressed by some 20%.

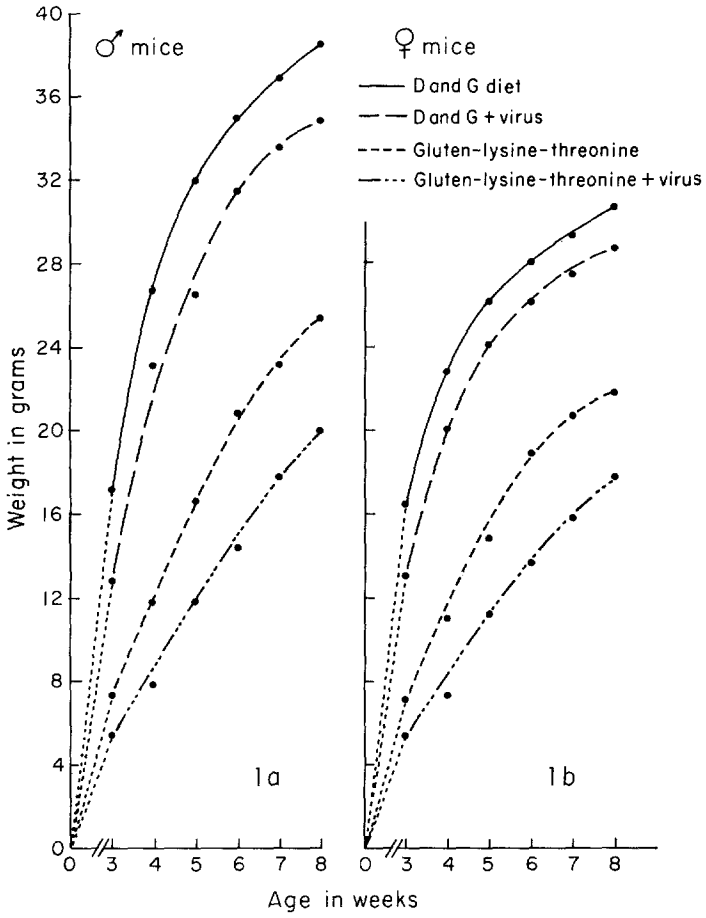


FIG. 1. Weight curves of COBS mice on two different diets; approximately 20 mice per group, each derived from 5 litters. Males (1*a*), Females (1*b*).

It is worth noting in Figs. 1 *a* and 1 *b* that, both in males and in females, the weight depressions caused by enterovirus infection are much more profound in the progeny of animals fed the gluten-lysine-threonine diet than in the progeny of animals fed the D&G pellets.

*Utilization of* $^{14}C$*-Actate or* $^{14}C$*-Glucose.* Table III and Fig. 2 illustrates the comparative metabolic activities of experimental and control animals mea-

TABLE II

*Effect of Neonatal Infection, Early Malnutrition, and Neonatal Infection Combined with Early Malnutrition on the Physical Characteristics of Various Organs**

| | Controls | Neonatal infection | Early malnutrition | Infection and malnutrition |
|---|---|---|---|---|
| Body Wt (g) | 36.5 | 33.6 | 24.8 | 23.2 |
| Liver Wt (g) | 2.27 | 1.89 | 1.24 | 1.24 |
| Brain Wt (g) | 0.52 | 0.51 | 0.44 | 0.42 |
| Heart Wt (g) | 0.19 | 0.17 | 0.13 | 0.13 |
| Lung Wt (g) | 0.23 | 0.21 | 0.15 | 0.18 |
| Spleen Wt (g) | 0.14 | 0.11 | 0.11 | 0.08 |
| Bone Wt (g) | 0.04 | 0.04 | 0.03 | 0.03 |
| Bone Length (cm) | 1.9 | 1.9 | 1.7 | 1.8 |

* Averages for female mice 3–5 months old, approximately 20 per group, each group from 5 different litters.

TABLE III

*Utilization of $^{14}C$-Acetate*

| Time after injection of $^{14}C$-acetate | Controls | Neonatal infection | Early malnutrition | Infection and malnutrition |
|---|---|---|---|---|
| (*hr*) | Respiratory $CO_2$ (cpm of $^{14}CO_2$/mmole $CO_2$) | | | |
| 0–½ | 14629 | 3998 | 3902 | 3317 |
| | (79.2%) | (73.5%) | (74.0%) | (70.5%) |
| ½–1 | 2849 | 913 | 732 | 772 |
| | (15.4%) | (16.7%) | (13.9%) | (16.4%) |
| 1–2 | 629 | 280 | 351 | 379 |
| | (3.4%) | (5.1%) | (6.7%) | (8.0%) |
| 2–3 | 202 | 153 | 164 | 147 |
| | (1.1%) | (2.8%) | (3.1%) | (3.1%) |
| 3–5 | 166 | 106 | 118 | 92.5 |
| | (0.9%) | (0.9%) | (2.2%) | (2.0%) |
| Total | 18475 ± 2339* | 5451 ± 753‡ | 5268 ± 775‡ | 4708 ± 542‡ |

Altogether, six male mice per experimental group and six control males, 2½ months old, received each 33 μCi/100 g body wt of 0.1 M sodium acetate-1-$^{14}C$ by the interperitoneal route. The samples of expired carbon dioxide were collected at indicated times after injection. The parentheses indicate the percentage of total $^{14}CO_2$ expired.

* Mean of respective group ± standard error of mean.

‡ $P < 0.01$.

sured in terms of production of respiratory $^{14}CO_2$ from $^{14}C$-acetate. Within 1 hr after injection of $^{14}C$-acetate, 94.6% of total respiratory $^{14}CO_2$ was expired; its production decreased rapidly thereafter. The expiration of total respiratory $^{14}CO_2$ was decreased by more than 70% in all experimental groups in comparison with the control group. As in the control group, most of the $^{14}CO_2$ (86.9–90.2%) was expired within 1 hr after injection of the $^{14}C$-acetate.

Table IV and Fig. 3 illustrate the comparative metabolic activities of experimental and control groups with regard to utilization of $^{14}C$-glucose. In contrast to the rapid metabolic turnover observed in the case of the acetate, glucose was metabolized much more gradually. During the 1st ½ hr, only 40.1% of the total $^{14}CO_2$ was expired in mice receiving $^{14}C$-glucose, as compared with 79.2% for the $^{14}C$-acetate group. The total respiratory $^{14}CO_2$ in the groups exposed to neonatal infection or early malnutrition was not significantly different from what it was in the conrrol group; however, it was greatly decreased in mice that had experienced both neonatal infection and early malnutrition.

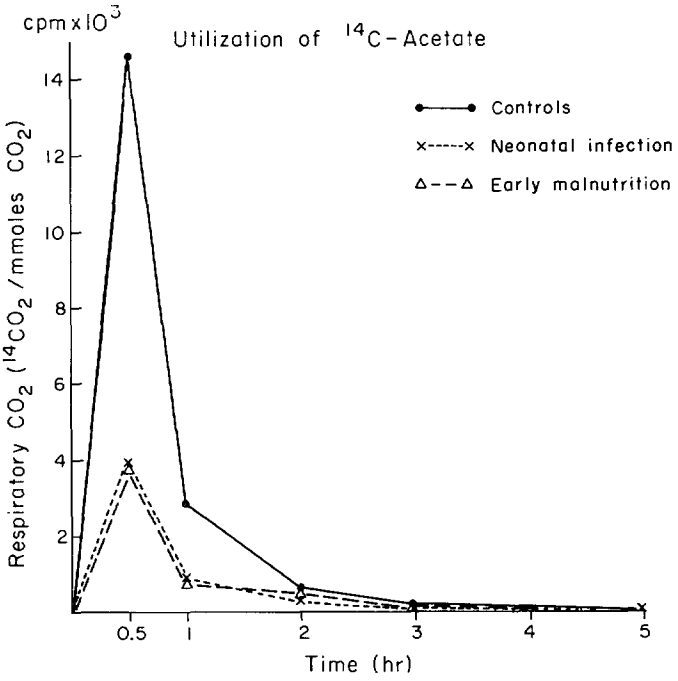


FIG. 2. See Legend for Table III.

The pattern of $^{14}C$-glucose utilization was different in infected mice from what it was in those of the malnutrition group. The infected group released 43.6% of the total respiratory $^{14}CO_2$ in the 1st ½ hr after glucose administration, whereas the malnutrition group released only 23.0%. During the 2nd ½ hr, $^{14}CO_2$ production fell to 22.3% in the infected group whereas it increased to 39.3% in the malnutrition group. The pattern of glucose metabolism in mice exposed to both neonatal infection and early malnutrition was essentially the same as in mice exposed only to early malnutrition.

As seen in Tables I and II, neonatal infection with the enterovirus and early malnutrition depressed body weight to approximately the same extent. The

mechanism of action may have been different, however, since the infected mice differed markedly from those nutritionally deprived with regard to the metabolic turnover of glucose. In animals subjected to both neonatal infection and early malnutrition, glucose metabolism reflected chiefly the nutritional effects. On the other hand, the combined effects of infection and malnutrition resulted in an impairment of glucose metabolism which was not apparent in animals exposed only to early malnutrition.

*Incorporation of $^{14}$C-Acetate or $^{14}$C-Glucose into Total Lipids of Various Tissues.*—Table V shows the incorporation of $^{14}$C-acetate and $^{14}$C-glucose into total

TABLE IV

*Utilization of $^{14}$C-Glucose*

| Time after injection of $^{14}$C-acetate | Controls | Neonatal infection | Early malnutrition | Infection and malnutrition |
|---|---|---|---|---|
| (*hr*) | Respiratory $CO_2$ (cpm of $^{14}CO_2$/mmole $CO_2$) | | | |
| 0–½ | 1481 | 1226 | 727 | 507 |
| | (40.1%) | (43.6%) | (23.0%) | (24.6%) |
| ½–1 | 937 | 626 | 1241 | 782 |
| | (25.3%) | (22.3%) | (39.3%) | (38.0%) |
| 1–2 | 724 | 574 | 689 | 509 |
| | (19.5%) | (20.4%) | (21.8%) | (24.9%) |
| 2–3 | 343 | 253 | 359 | 169 |
| | (9.3%) | (9.0%) | (11.4%) | (8.2%) |
| 3–5 | 214 | 128 | 144 | 92.1 |
| | (5.8%) | (4.6%) | (4.6%) | (4.5%) |
| Total | 3701 ± 267* | 2806 ± 130 | 3160 ± 339 | 2059 ± 153‡ |

Six male mice per experimental group and six control males, 2½ months old, each received 33 μCi/100 g body weight of 0.55 M glucose-UL-$^{14}$C by the intraperitoneal route. The samples of expired carbon dioxide were collected at indicated times after injection. The parentheses indicate the percentage of total $^{14}CO_2$ expired.

* Mean of respective group ± standard error of mean.

‡ $P < 0.01$.

lipids of liver and brain. The acetate incorporation was higher in liver than in brain, but no difference could be recognized between these two organs with regard to glucose incorporation. The ability to incorporate acetate into total lipids of liver and brain was significantly reduced in all experimental groups, but the incorporation of glucose was not affected in a detectable manner.

## SUMMARY

Specific pathogen-free mice were exposed to three different kinds of environmental stress during early life: (*a*) by infecting them with a mouse enterovirus on the second day after birth; (*b*) by placing the mother during pregnancy and lactation on a mildly deficient diet containing wheat gluten supplemented with

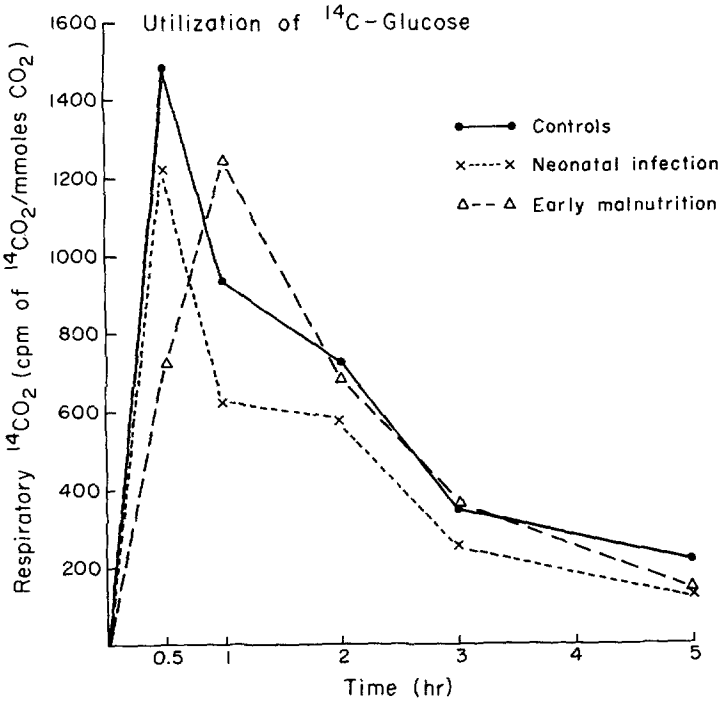


FIG. 3. See Legend for Table IV.

TABLE V

*Incorporation of $^{14}C$-Acetate or $^{14}C$-Glucose into Total Lipids of Liver and Brain**

| | Controls | Neonatal infection | Early malnutrition | Infection and malnutrition |
|---|---|---|---|---|
| | Incorporation of $^{14}C$-Acetate (cpm/mg total lipids) | | | |
| Liver | 86.8 ± 2.74 | 55.4 ± 1.73‡ | 44.3 ± 7.57‡ | 32.4 ± 6.65‡ |
| Brain | 44.0 ± 7.30 | 21.6 ± 5.70§ | 23.5 ± 5.72§ | 21.2 ± 3.93§ |
| | Incorporation of $^{14}C$-Glucose (cpm/mg total lipids) | | | |
| Liver | 30.2 ± 6.18 | 26.5 ± 5.06 | 33.7 ± 6.88 | 23.8 ± 4.31 |
| Brain | 27.1 ± 2.48 | 31.0 ± 5.48 | 31.1 ± 3.52 | 28.8 ± 3.95 |

* Average for 6 males per group, 2½ months old. Mean of respective group ± standard error of mean.

‡ $P < 0.01$.

§ $P < 0.05$.

small amounts of lysine and threonine; (*c*) by combining *a* (neonatal infection) and *b* (early malnutrition).

All animals survived the three types of stresses, but all exhibited marked depressions of metabolic activity, and of body weights and organ weights.

These depressions lasted throughout the experimental period even though all animals were placed under optimum conditions of nutrition and husbandry after weaning, and maintained under these same conditions thereafter.

Metabolic activity was determined by measuring the turnover of $^{14}C$-acetate and $^{14}C$-glucose in respiratory $CO_2$, and their incorporation in total lipids of liver and brain.

The utilization of $^{14}C$-acetate was profoundly depressed in all experimental groups with regard to both elimination in respiratory $CO_2$ and their incorporation in total lipids of liver and brain.

In contrast, the utilization of $^{14}C$-glucose was much less affected; its incorporation into lipids was not decreased and its elimination in respiratory $CO_2$ was depressed only in animals having experienced both neonatal infection and early malnutrition.

The extent of weight depression per 100 g of body weight differed according to the organ and the type of stress. Irrespective of the organ, however, depression of weight was largest in animals having experienced both neonatal infection and early malnutrition. And irrespective of the type of stress, the brain exhibited the smallest depression of weight relative to total body weight.

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