# DNA (Cell Number) and Protein in Neonatal Rat Brain: Alteration by Timing of Maternal Dietary Protein Restriction [1]

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*ABSTRACT* Pregnant rats were fed a protein-free diet during five periods of pregnancy: days 0 to 10, 10 to 15, 13 to 18, 15 to 20 or 10 to 20, and normal diet during the remaining time. In the case of the days 0 to 10 group, 62% of the females failed to litter. There was no significant change in the amount of food intake of pregnant animals in any of the groups; yet in all cases there were significant decreases in body weights, cerebral weights, cerebral DNA (cell number) and cerebral protein of the neonatal animals even though, until day 15, the total protein increment of the embryo and its supporting tissue constitutes only an insignificant fraction of the average maternal protein intake. An explanation of the neonatal underdevelopment, involving the triggering of a hormonal mechanism and resulting in a placental deficiency has been proposed. After day 15, this mechanism may be supplemented by an actual deficiency of the amino acids required for fetal protein synthesis. J. Nutr. *101:* 1265–1270, 1971.

In the preceding papers of this series (1, 2), it has been demonstrated that, in rats, maternal protein restriction (8%) 1 month prior to mating and throughout pregnancy resulted in a significant decrease in the following neonatal parameters: body weight, cerebral weight, cerebral DNA (total cell number), and cerebral protein. Similar results were obtained in other laboratories: Chow and his collaborators (review in (3)) have demonstrated that 50% dietary restriction during pregnancy and lactation produced permanently growth-stunted offspring, but the effect on cerebral DNA was not investigated. Winick (4) has demonstrated that this decrease in cerebral DNA can be made much more pronounced by postnatal dietary restriction before weaning. Zeman and her collaborators (5, 6) have also shown that the decrease in cerebral DNA and number of neurons can be demonstrated if maternal protein restriction is limited to pregnancy only. We have recently extended our studies to the newborns in the second generation (2).

In the present work we have investigated the effects, especially on brain development, of protein restriction of short duration during various periods of pregnancy. Because of the short duration, the protein restriction was made more severe, i.e., complete protein deprivation.

## EXPERIMENTAL

Albino rats used in the previous (1, 2) and present work are Sprague-Dawley derived; these rats have now been bred in our laboratory for 22 generations. Virgin females 3 months old and weighing 200 to 260 g were mated; the presence of a vaginal plug was considered day 0 of pregnancy. The animals were fed a pelleted diet containing 20.5% protein [2] except for the experimental animals during the periods of total protein deprivation: days 0 to 10, 10 to 15, 13 to 18, 15 to 20 or 10 to 20. During these periods the pelleted diet was

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[2] Pelleted diet (2882 calories/1000 g) was Wayne Mousebreeder Block, supplied by Allied Mills, Chicago, Ill.

replaced by the powdered protein-free diet.[3] Upon completion of this period, the experimental animals were returned to the normal pelleted diet.

Food intake of normal or protein-free diet per 24 hours was measured for all animals, by feeding known amounts and weighing the remainder (including any spilled food).

After delivery, the mothers were dissected and their uteri examined for resorption sites. The newborns were weighed and decapitated within 6 hours after delivery. The brains (cerebral hemispheres, without cerebellum and olfactory lobes) were immediately removed and weighed; they were then frozen and subsequently used for analysis. DNA was determined by a modification of the diphenylamine colorimetric method (7, 8); protein was determined by a modification of the Lowry colorimetric method (9).

In order to determine the immediate effects of protein deprivation, caesarians were performed 1 or 5 days after the period of deprivation. The special aim was to examine placental as well as uterine development. The period of deprivation between days 10 and 15 was most suited for this purpose since meaningful and reproducible data can be obtained from a 16-day caesarian, as well as performing caesarians on day 20 to demonstrate the effects of refeeding for the remaining 5 days. Eight experimentals and eight controls were weighed, and caesarians performed, using ether anesthesia, at the standard time (2 PM). The uteri containing the fetuses were dissected free of connective tissues and weighed. The fetuses were then removed and their respective placentas dissected free of all membranes, blotted, and weighed. The remaining uterine tissue as well as membranes were reweighed and the weight of the amniotic fluid determined. The placentas were dried to a constant weight and their water content determined.

## RESULTS

Table 1 represents the effects of short-term total protein deprivation on the food intake of the pregnant rat itself. Column 1 shows the period, counting from positive mating, in which the diet was completely protein-free (PF); during the remainder of the pregnancy the diet had normal (N) protein content. Protein deprivation of such a short duration had some effect on the food intake of the pregnant animals. Still, as can be seen in table 1, regardless of the diet, the food intake per 24 hours of experimental and control animals was within the normal range. Thus, the total protein intake of the experimental animals during the entire pregnancy was reduced as much as 50% as in the case of the last group. The experimental animals did not consume more food either during or after the period of protein deprivation.

Table 2 represents the effects of the protein deprivation on the offspring of these animals. Normal controls were always available for concurrent comparisons with protein-depleted animals; there were no

[3] Powdered protein-free diet (2675 calories/1000 g) was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio; it had the following composition: (in %) corn starch, 70; Alphacel (cellulose), 15; vegetable oil, 10; salt mixture, USP XIV, 4; cod-liver oil, 1. This diet was supplemented with vitamin B complex (Becotin, Lilly), consisting of: (in mg) thiamin, 50; riboflavin, 50; pyridoxine, 20.5; niacinamide, 250; pantothenic acid, 125; and $B_{12}$, 5 μg, per 1000 g. Biotin, folate and choline were not added to the diet but no evidence of deficiency of these vitamins was seen in these short experiments.

TABLE 1

*The effects of protein-free diet on the food intake of pregnant rats*

| Period of protein deprivation [1] | ♀ Number mated | Entire food intake [2] | | | | Protein intake total pregnancy |
|---|---|---|---|---|---|---|
| | | PF | N | Total | | |
| | | *g* | *g* | *g* | *kcal* | *g* |
| None (controls) | 27 | — | 300±32 | 300±32 | 865±92 | 61.5±6.5 |
| 0–10 | 13 | 107± 6 | 162±10 | 269±12 | 753±34 | 33.2±2.1 |
| 10–15 | 6 | 63± 4 | 204±13 | 267±18 | 757±50 | 41.8±2.8 |
| 13–18 | 5 | 72± 6 | 218±17 | 290±12 | 819±36 | 44.6±3.5 |
| 15–20 | 5 | 60± 5 | 203±18 | 263±21 | 746±60 | 41.6±3.7 |
| 10–20 | 10 | 123±11 | 147±13 | 270±17 | 753±49 | 30.1±2.7 |

[1] Days after mating (vaginal plug).
[2] For total pregnancy; mean ± SD. PF, protein-free during period of deprivation; N, normal, during nondeprived period.

TABLE 2

*The effect of protein-free diet on the offspring*

| Period of protein deprivation [1] | Numbers | | | | Offspring [3] | | | |
|---|---|---|---|---|---|---|---|---|
| | Mothers | | Offspring [2] | | | Cerebrum | | |
| | Mated | Littered | Living per litter | % Stillborn | Body weight | Weight | DNA | Protein |
| | | | | | *g* | *g* | *μg* | *mg* |
| None (controls) | 27 | 26 | 10.0 | 2 | 6.2 ± 0.5 [3] | 0.1718 ± 0.0107 | 585 ± 28 | 9.33 ± 1.14 |
| 0–10 | 13 | 5 | 10.6 | 7 | 5.9 ± 0.5 [b] | 0.1671 ± 0.0182 | 552 ± 38 [a] | 8.50 ± 1.40 [a] |
| 10–15 | 6 | 6 | 8.7 | 7 | 5.8 ± 0.5 [a] | 0.1561 ± 0.0108 [a] | 541 ± 34 [a] | 8.06 ± 0.83 [a] |
| 13–18 | 5 | 4 | 9.3 | 18 | 5.4 ± 0.6 [a] | 0.1620 ± 0.0167 [b] | 587 ± 34 | 7.69 ± 0.54 [a] |
| 15–20 | 5 | 3 | 10.0 | 9 | 5.4 ± 0.8 [a] | 0.1521 ± 0.0180 [a] | 543 ± 23 [a] | 8.13 ± 0.67 [a] |
| 10–20 | 10 | 10 | 9.0 | 8 | 4.6 ± 0.9 [a] | 0.1497 ± 0.0198 [a] | 522 ± 37 [a] | 8.10 ± 0.91 [a] |

[1] Days after mating.
[2] Neonatal examination.
[3] Each value represents the mean ± sd. [a] Significant at $P < 0.001$ level. [b] Significant at $0.01 > P > 0.001$ level.

significant differences between the controls for any of the parameters measured. Protein deprivation started before implantation (days 0 to 10) resulted in an increased failure to implant or early resorptions as demonstrated by the fact that 62% of the positively mated females failed to litter. Subsequent examination of their uteri at term failed to show resorption sites. (The number of offspring per litter was normal in those animals that littered.) In the remaining animals, the number of offspring per litter was also normal, but the number of stillborns was substantially increased, especially when the protein deprivation was between days 13 and 18.

It can be seen that regardless of when protein deprivation occurred, the newborns had (with two exceptions) significant decreases in body weight, cerebral weight, cerebral DNA (related to cell number, see Discussion) and cerebral protein. In general, the decreases tended to be larger if the period of deprivation was longer and if it was imposed later during pregnancy. In the 16-day caesarians, the average wet placental weight for the controls was 0.3398 ± 0.0695 g (n = 38) compared to 0.2537 ± 0.0460 g (n = 45) for the corresponding experimental animals, a 25% reduction. A similar reduction (28%) was observed in the average dry placental weight: Control, 0.0534 ± 0.0119 g (n = 38); experimental, 0.0387 ± 0.0069 g (n = 45). In spite of refeeding for the remaining 5 days, the 20-day caesarians still showed a 14% reduction in the wet placental weight: Control, 0.5882 ± 0.1610 g (n = 22); experimental, 0.5032 ± 0.0618 g (n = 35). The reduction in the dry placental weight was 12%: Control, 0.0954 ± 0.0289 g (n = 22); experimental, 0.0836 ± 0.0161 g (n = 35). The placental water content was the same for experimentals and controls at both times. In the 20-day caesarians the amount of amniotic fluid calculated per newborn was 1.08 ml in the controls as compared to 0.93 ml in the experimentals, representing a reduction of 14%. The calculated uterine weight per newborn showed a 37% reduction: Control, 1.04 g; experimental, 0.66 g. As can be seen, all animals which were deprived from day 10 to 15 of pregnancy demonstrated a nonrepairable impairment in spite of refeeding for the remaining 5 days.

## DISCUSSION

It appeared of interest to relate the protein content of fetuses and their supporting tissues (uterus and placentas) at any particular period of gestation, to the normal maternal protein intake for this period.

Ten fetuses (an average litter size) and their supporting tissues have a protein content of 620 mg by day 15 (calculated from literature (10–13) and our own data). They constitute only 1.3% of the total protein intake of a control pregnant female for the same time period (46.1 g). The protein content of these tissues increases by 2330 mg between days 15 and 20, representing 15% of the total protein intake of a control female during these 5 days.

Protein deprivation, even of the shortest duration tested, did not fail to produce

significant effects in the offspring (table 2). Of these, the finding that the deprivation of protein (14) or an essential amino acid (13) before implantation, produces failure to litter, has been previously reported. This failure was reported to be due to early resorptions (around day 10) which, as in our case, were often not detectable at the end of pregnancy. The failure was traced to faulty implantation and placentation, caused by a lack of estrogen and progesterone (12, 15–19), in turn caused by a lack of pituitary gonadotropins (20), especially prolactin (17, 21). These investigators were mainly interested in maintenance or nonmaintenance of pregnancy; brain development was not studied.

In the present work, we were especially interested in the effects on brain development.

Previous work in our laboratory (1, 22–24) has demonstrated that individual brain weights cannot be used as a meaningful parameter of brain development (DNA); nevertheless, for a sufficiently large population we have demonstrated that, on the average, a higher number of neonatal brain cells necessitates a higher neonatal brain weight (25). The determination of neonatal brain DNA is a convenient and objective quantitative method for determination of total neonatal brain cell numbers; such a determination is based on the findings that normal neuron and glial cells at birth are essentially diploid and that the amount of DNA per diploid cell of a given species is constant (reviews in (23, 24)). The total amount of brain DNA in the developing rat reaches a transient plateau at birth; at the plateau this amount is one of the most constant parameters of the developing organism (22–24).

As shown in table 2, protein deprivation even of the shortest duration, produced significant decreases in neonatal body weight, cerebral weight (with one exception), cerebral DNA (with one exception), i.e., cerebral cell number, and cerebral protein. These differences were observed in all periods of protein deprivation. In accordance with the above mentioned constancy of normal neonatal cerebral DNA content, the differences in DNA content were (with one exception) more conservative than the differences in other parameters. As expected, differences in cerebral protein content were higher than differences in cerebral weight and cerebral DNA (cell number); this latter result indicates that the protein content per cell was also lower than normal.

As can be seen, significant effects on brain development were obtained even in the periods (0 to 10 and 10 to 15 days) when the total protein increments of the embryo (fetus) and its supporting tissues constituted only an insignificant fraction of average protein intake. Thus, the observed effects are unlikely to be due to an actual deficiency of amino acids per se as required for embryonal protein synthesis. This is especially true for the period before implantation (days 0 to 6). As mentioned above, the disturbance was traced to deficient placental development caused by a deficiency in estrogen and progesterone, in turn caused by a deficiency in maternal pituitary gonadotropic hormones. The latter might presumably be triggered by a change in amino acid balance or serum proteins (12) acting in the pituitary and/or the hypothalamus [4] which produces pituitary hormones releasing factors. After day 11, when the placenta starts to assume the hormonal functions of the maternal pituitary, the deficiency in placental development may further contribute to the overall effect (compare also (19)). It is of interest that Winick also demonstrated the adverse effects of maternal protein restriction on placental development as early as on day 13 (4).

Our data on the 16- and 20-day caesarian following deprivation from days 10 to 15, also indicate deficient placental development (see Results). This deficiency persists to term (20-day caesarian).

After day 15, the total protein increments of the fetus and its supporting tissue cease to be insignificant. Brain underdevelopment due to protein deprivation after this time may be due to a direct deficiency of amino acids required for protein synthesis. The adverse effect on the development of the placenta may also continue. The most pronounced effects were

[4] Negro-Vilar, A., E. Dickerman and J. Meites 1968 Effects of starvation on pituitary FSH and hypothalamic FSH-releasing factor (FSH-RF) in male rats. Federation Proc. 27: 269 (abstr.).

obtained by protein deprivation from days 10 to 20. This indeed seems to indicate the cumulative effect of both these mechanisms. Obviously, more work is required before these views may be fully accepted.

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