Maternal vitamin A restriction alters biochemical development of the brain in rats

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Summary. The biochemical development of the fetal brain in relation to maternal vitamin A restriction was studied in rats. The vitamin A status of pregnant rats was varied by supplying low, medium and adequate amounts (6, 40, and 100 µg retinol/day/kg body weight, respectively) of vitamin A during pregnancy and suckling. The maternal vitamin A restriction caused an altered brain development in terms of tissue weight, DNA, RNA and protein levels, and biosynthesis of DNA and protein from [^{3}H]-thymidine and [^{3}H]-leucine, respectively. A dose-dependent effect of maternal vitamin A restriction on the metabolism of DNA, RNA and protein was noticed in the developing fetal brain of rats.

Key words. Brain development; DNA; RNA; protein; vitamin A; rat.

Biochemical development in mammals depends upon a series of subtle alterations in cell-controlled mechanisms. The multitude of regulatory factors which control the complex flow of nutrients to the site of synthesis of cell components is not yet well elucidated. Normal growth of organs has been studied by several workers by employing the DNA, RNA and protein levels of the tissue as biochemical parameters of growth and development[1-3]. Winick and Noble[1] have suggested that organ growth is accomplished in three phases: 1) a period of rapid cell division, i.e. increase in DNA content with no change in the weight-to-DNA ratio (hyperplasia); 2) cell division continues slowly but cells increase in size, i.e. there is an increase in the weight-to-DNA ratio with increase in DNA content (combined hyperplasia and hypertrophy); and 3) cell division stops and growth continues by enlargement of cells, i.e. there is an increase in the weight-to-DNA ratio with no change in total DNA content (hypertrophy).

Involvement of vitamin A in fetal growth and development has been reported ever since the discovery of the vitamin[4-6]. Vitamin A deficiency in the children of socio-economically depressed populations is quite common in many areas of the world. Studies in rats have shown that severely vitamin A deficient females fail to conceive and partially-deficient females conceive but usually abort or resorb the fetus in later gestation[5]. Under conditions of vitamin A deficiency malformed fetuses have been observed. It has been suggested that fetal malformations resulting from maternal vitamin A deficiency are determined during the period of active organogenesis[5,6]. Dobbing[7] has demonstrated that there is a specific period of brain growth during which the organ is susceptible to growth-restriction resulting in a permanent physical deficit. Maternal hypo- and hyper-vitaminosis A have been reported to influence the growth of several organs in terms of DNA, RNA and protein contents[8-10]. No information is available yet regarding the effects of maternal vitamin A deficiency on the metabolism of DNA, RNA and protein in the brain of developing rats. Since a model with total vitamin A deficiency cannot be used to study the growth and development of the fetus, for obvious reasons, the present study was conducted to elucidate the influence on the developmental pattern of DNA, RNA and protein in the rat brain of giving a low (6 µg), medium (40 µg), or adequate (100 µg) supply of vitamin A to vitamin A-deprived dams.

Materials and methods

Wistar strain female rats (160–180 g) from the colony maintained in the Institute were kept on a vitamin A deficient diet[11] for a period of one week. The rats were housed individually with free access to water. The hepatic and plasma vitamin A stores of these partially vitamin A-depleted females, prior to vitamin A supplementation, were 27.52 ± 2.08 µg/g liver and 15.12 ± 1.62 µg/dl plasma respectively. On the 8th day of feeding, these females were mated with normal male rats of the same strain. The period of conception was determined by the presence of sperm in the vaginal smear and this day was taken as day 0 of gestation. The pregnant rats were divided into three groups and pair-fed a vitamin A deficient diet with varying amounts of vitamin A supplementation as follows:

Group L: Low vitamin A (6 µg/day/kg b.wt);
Group M: Medium vitamin A (40 µg/day/kg b.wt);
Group A: Adequate vitamin A (100 µg/day/kg b.wt).

Three dams from each group, and their litters, were sacrificed on the 20th day of gestation, at birth, and on the 10th and the 23rd day of postnatal age. 3 h before sacrifice on the 20th day of gestation each dam, and at birth and at days 10 and 23 of postnatal age each pup, was injected i.p. with a sterile saline solution containing [5′-^{3}H]-thymidine (sp. act. 15.2 Ci/mmole) or DL[4,5-^{3}H]-leucine (sp. act. 12 Ci/mmole), 10 µCi/100 g b.wt in separate experiments. Dams were sacrificed by decapitation

and litters were collected surgically on the 20th day of gestation. Pups were sacrificed by cervical dislocation and their brains were quickly removed, immersed in ice-cold physiological saline (0.9% w/v), cleaned, pooled (3–4 in one sample) and processed for the isolation of DNA, RNA and protein.

A 10% (w/v) homogenate of the tissue in ice-cold distilled water was prepared and DNA and RNA were extracted according to the method of Munro and Fleck[12]. Proteins were isolated using 10% trichloroacetic acid and were delipidized with an ethyl alcohol: diethyl ether (2:1, v/v) mixture. DNA was estimated by the diphenylamine reaction[13], RNA by the orcinol reaction method[14] and proteins were quantitated by the method of Lowry et al.[15]. Suitable aliquots of DNA and protein extracts were taken in scintillation vials for radioactivity counting in a Kontron MR-300 liquid scintillation counter using a dioxane-based scintillation cocktail.

The results were analysed statistically and the significance of differences was calculated using Student's t-test.

Results and discussion

a) *Tissue weight.* The tissue weight increased linearly with the age of the pups. No significant effect of the maternal vitamin A restriction on the brain weight was noticed among the three groups during pre- and postnatal development of pups (table 1). It is likely that during gestation the vitamin A required for development of the brain was being supplied by mobilization of maternal reserves through the placenta, as the dams used were only partially vitamin A deficient. It may be stated that, in spite of the reduced vitamin A intake in the L and M groups, the mothers' plasma and placental vitamin A contents were comparable in the three groups during pregnancy[16].

b) *DNA, RNA and protein levels.* The levels of DNA and RNA in the pups' brains increased almost linearly up to 10 days of age and then reached a plateau in groups L and M; whereas, in group A, DNA and RNA contents continued to increase (table 1). The developmental pattern of proteins was somewhat different, as it increased linearly with the age of the pups in all groups (table 1). Like the tissue weight, the pre- and postnatal increase was linear for brain proteins. The dietary vitamin A status of the mother profoundly affected the DNA, RNA and protein contents in the developing brain mainly during postnatal growth. The brain protein content was not significantly influenced by the maternal vitamin A restriction on the 10th day of age; however, it was significantly decreased in group L as compared to group A at day 23 of postnatal age (table 1). These results clearly indicate the vitamin A dependence of the metabolism of DNA, RNA and protein in the developing brain. The significant decrease observed in DNA, RNA and protein levels of brain during its postnatal development could be ascribed to the low hepatic vitamin A stores of fetuses. We have observed earlier that the vitamin A reserves in the livers of pups derived from dams supplemented with a low level of vitamin A were remarkably small in comparison to those receiving on adequate vitamin A supplement[16, 17].

c) *Cell number and cell size.* The developmental pattern of cells (cell number) showed a steady increase with the age of pups in all groups, but the magnitude of increase was significantly affected by the dietary vitamin A status of the mother (table 2). In contrast to the cell number, cell size in brain decreased gradually with the age of pups (table 2). No significant effect of the maternal vitamin A restriction on the cell size was noticed during prenatal development of pups. These results thus indicate the necessity of vitamin A for the growth of brain cells predominantly during postnatal development.

d) *Incorporation of labelled precursors into DNA and protein.* The decreased amounts of DNA, RNA and protein in the brains of pups from mothers maintained on low supplementation of vitamin A, at various periods

Table 1. Effect of maternal vitamin A restriction on tissue weight, DNA, RNA and protein contents of the brain of rats at various periods of development

Period	Parameter	Dietary groups L	M	A
20th day of gestation	Tissue weight (g)	0.12 ± 0.02	0.14 ± 0.01	0.18 ± 0.02
	DNA (mg/organ)	0.04 ± 0.008	0.06 ± 0.007	0.05 ± 0.004
	RNA (mg/organ)	0.21 ± 0.02[a]	0.24 ± 0.03	0.26 ± 0.04
	Protein (mg/organ)	2.32 ± 0.25	2.28 ± 0.33	2.46 ± 0.48
Birth	Tissue weight (g)	0.24 ± 0.03	0.35 ± 0.05	0.37 ± 0.04
	DNA (mg/organ)	0.40 ± 0.02[a, b]	0.57 ± 0.03	0.69 ± 0.05
	RNA (mg/organ)	0.45 ± 0.04[a, b]	0.72 ± 0.06	0.74 ± 0.06
	Protein (mg/organ)	9.75 ± 1.02[a, b]	14.38 ± 1.12	15.09 ± 1.48
10th day postnatal	Tissue weight (g)	0.65 ± 0.07	0.73 ± 0.05	0.82 ± 0.11
	DNA (mg/organ)	1.34 ± 0.06[a]	1.62 ± 0.11[a]	2.08 ± 0.17
	RNA (mg/organ)	1.68 ± 0.15[a, b]	2.58 ± 0.21	2.67 ± 0.29
	Protein (mg/organ)	38.27 ± 3.28	45.17 ± 6.23	51.37 ± 5.82
23rd day postnatal	Tissue weight (g)	1.12 ± 0.08	1.21 ± 0.11	1.30 ± 0.09
	DNA (mg/organ)	1.38 ± 0.10[a]	1.76 ± 0.14[a]	2.60 ± 0.09
	RNA (mg/organ)	1.97 ± 0.17[a, b]	2.77 ± 0.22[a]	3.88 ± 0.31
	Protein (mg/organ)	118.39 ± 10.32[a]	138.54 ± 12.98	159.34 ± 16.05

Values are mean ± SEM from three litters in each group. [a] and [b] show statistically significant values ($p \leq 0.05$) [a] vs A group; [b] vs M group.

Table 2. Developmental pattern of cells in brain tissue in relation to maternal vitamin A status

Period	Parameter	Dietary groups L	M	A
20th day of gestation	Cell number	5.97 ± 1.29	8.71 ± 1.45	7.26 ± 0.32
	Cell size	6.92 ± 0.34[a,b]	4.69 ± 0.48	4.32 ± 0.25
Birth	Cell number	18.87 ± 1.03[a,b]	30.26 ± 2.03	32.39 ± 3.93
	Cell size	3.76 ± 0.35[a,b]	2.50 ± 0.18	2.00 ± 0.18
10th day postnatal	Cell number	215.97 ± 9.52[a]	259.35 ± 17.42	335.65 ± 28.25
	Cell size	3.51 ± 0.19[a]	3.03 ± 0.25[b]	2.29 ± 0.09
23rd day postnatal	Cell number	205.85 ± 17.59[a,b]	282.91 ± 22.09[a]	426.29 ± 18.19
	Cell size	3.02 ± 0.19	4.21 ± 0.36	3.13 ± 0.26

Values are mean ± SEM from three litters in each group. Cell number is expressed as number of nuclei × 10^{-6}. Cell size is expressed as weight/nucleus (ng). [a]and [b] show statistically significant values ($p \leq 0.05$). [a]vs A group; [b]vs M group.

of development, could be either due to the decreased synthesis of these substances, their increased breakdown, or both. To obtain an insight into these possibilities, the incorporation of [^{3}H]-thymidine into DNA and [^{3}H]-leucine into the proteins of the developing fetal brain was studied and the results are shown in figures 1 and 2. The incorporation was expressed as total activity (dpm/organ) to assess the retention of label and hence the synthesis of DNA or protein, and also as specific activity (dpm/mg DNA or protein) to know their turnover (net synthesis) for comparative purposes. The developmental pattern of incorporation of these precursors into DNA or protein (dpm/organ) was nearly similar to that observed for their amounts (figs 1, 2). The DNA synthesis and its turnover was significantly reduced in the pups derived from group L as compared to those from group A at all periods of development studied (fig. 1). Unlike DNA, the turnover of brain proteins decreased until the 10th day of age in all three groups and thereafter it remained nearly constant (fig. 2), indicating an enhanced rate of catabolism during gestation and suckling. Furthermore, protein synthesis from [^{3}H]-leucine was significantly reduced in the brains of pups derived from dams given low and medium vitamin A supplements, as compared to adequately-supplemented dams, at 23 days of age.

Our results thus demonstrate that the growth of the brain and the metabolism of DNA, RNA and protein appear to be dependent on the availability of vitamin A to the dams during pregnancy and suckling. The variations noted in DNA, RNA and protein levels of the brain during postnatal development are likely to be due to differences in fetal hepatic vitamin A stores [17]. The hepatic reserves of pups derived from groups L and M were significantly low as compared to those from group A owing to the low maternal vitamin A status and/or low availability of vitamin A from the milk and further because the diet available for nibbling was vitamin A free. The literature available on fetal malformations due to maternal vitamin A deficiency and the results of the present study provide further evidence for the action of vitamin A in cellular differentiation and growth; however, the exact mecha-

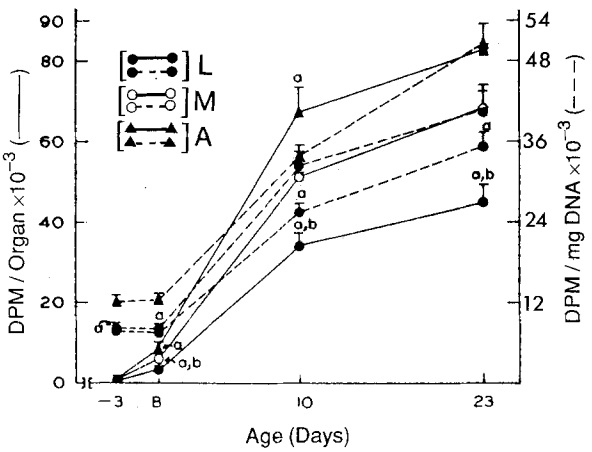

Figure 1. Incorporation of [^{3}H]-thymidine into the brain DNA of pups at various periods of development in relation to maternal vitamin A status.
Total activity is expressed as dpm/organ and the specific activity as dpm/mg DNA. Values are mean ± SEM from three litters in each group. Vertical bars represent the SEM. [a]and [b] show statistically significant values where $p \leq 0.05$. [a]vs A and [b]vs M.

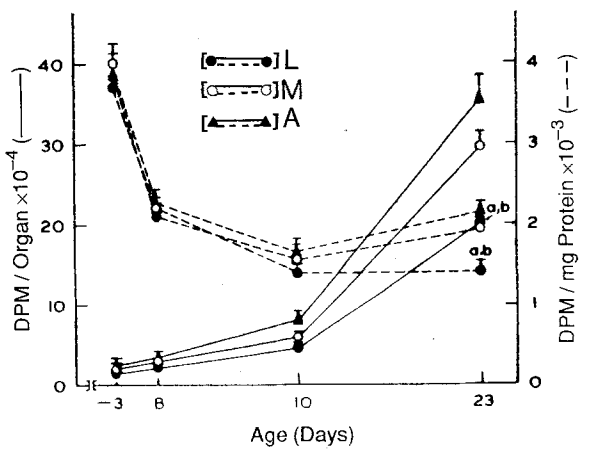

Figure 2. Incorporation of [^{3}H]-leucine into the brain proteins of pups at various periods of development in relation to maternal vitamin A status. Details are the same as described in figure 1.

nism of vitamin A action on brain maturation is not yet clear.

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Localisation of acetylcholinesterase in rat myotubes in the presence of β-endorphin and β-endorphin-(1-27)

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Summary. In rat embryo skeletal myotubes, acetylcholinesterase is present, as multiple forms, and can be detected in deposits at the cell surface. Myotubes cultured in the presence of β-endorphin, exhibit an increased predominance of the globular (precursor) forms of the enzyme, which are largely restricted to intracellular sites associated with nuclei. In the presence of β-endorphin-(1-27), the relative proportions of the different forms of acetylcholinesterase is similar to that seen in the controls, but the enzyme is intracellular and has a characteristic focal localisation in the myotube.
Key words. Acetylcholinesterase; myotubes; β-endorphin.

Acetylcholinesterase (AChE, EC 3.1.1.7) in skeletal muscle is under the trophic control of the motor nerve. Studies on developing myotubes in vitro have shown that both electromechanical activity [1, 2] and soluble factors [3, 4] can regulate the activity of the different molecular forms of the enzyme. β-Endorphin, a neuropeptide which coexists with acetylcholine in motoneurones of developing rodents [5, 6] can exert a regulatory influence over AChE, reducing the proportion of the asymmetric A_{12} form, and increasing that of the globular (G_1, G_2, G_4) forms of the enzyme [7]. β-Endorphin and its C-terminally-truncated derivative, β-endorphin-(1-27), are released by embryonic spinal cord in vitro [7]. In this report, we show that a striking redistribution of AChE accompanies the changes in enzyme activities in rat myotubes cultured in the presence of the peptides.

Myotubes were cultured from rat embryos of 20–21 days gestation, as previously described [7]. After fusion had occurred, the cells were cultured for a further 3 days and then transferred to a maintenance medium containing 0.25% chick embryo extract, 0.2 U/ml insulin, 50 μg/ml transferrin and 32 ng/ml progesterone. In some experiments the cells were exposed to β-endorphin or β-endorphin-(1-27) for up to 7 days. Cells were either harvested and the AChE molecular forms extracted and analysed as previously described [7], or they were stained histochemically for AChE using a modified direct colouring reaction [8, 9] in phosphate buffer (pH 7.5, reaction time 120 min) after the method of Koelle [10]. AChE was detected using acetylthiocholine iodide as substrate in the presence of 0.1 mM tetraisopropylpyrophosphoramide, and butyrylcholinesterase (BuChE) was detected using S-butyrylthiocholine chloride as substrate in the presence of 10 μM 5,5′-dithiobis(4-alkyldimethylammoniumphenyl)pentan-3-one dibromide (reaction time 5 h).

In control cultures, three major peaks of AChE were detected, of which a significant proportion of the enzyme was the asymmetric, A_{12} form (fig., A). In rat myotubes, this form is restricted in its localisation to the cell surface [9]. Most of the AChE was at or near the cell-surface (fig., B). In β-endorphin-exposed cultures the AChE activity was recovered principally as low and medium-sized AChE forms (fig., C) which are precursors to the A_{12} form. In these cells the AChE was seen to be present exclusively in the nuclear envelope (fig., D, arrowed) and Golgi apparatus (double-arrowed), and no cell mem-