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Postnatal Vascular Growth in the Neocortex of Normal and Protein-deprived Rats

Morphometric Studies

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Summary. The postnatal vascular growth in the neocortical area 18 of normal and pre- and postnatally protein-deprived rats was examined. For control rats the specific length, the specific surface and the volume fraction of vessels increased rapidly between 7 and 20 days of age. Thereafter, only a minor increase was seen. In protein-deprived rats there was no increase in the specific length of vessels between 7 and 10 days of age and this variable was still reduced at 30 days of age compared to controls. This reduction was due to a decrease in the specific length of thin vessels ($\varnothing < 8.25\,\mu$) whereas the specific length of wider vessels was not affected by the protein deprivation. There were no significant differences in the specific surface or volume fraction of vessels between control and protein-deprived rats. These findings indicate an adaptive increase in luminal diameter of vessels in the protein deprived rats during postnatal development. At 90 days of age no significant differences between vascular variables of control and protein-deprived rats were seen.

Key words: Rat – Protein deprivation – Neocortex – Vessels – Morphometry

Experimental protein deficiency may be achieved in animals either by reducing the amount of available food (protein-calorie malnutrition) or by decreasing the protein content in diets given ad libitum (protein deprivation). These conditions have been shown to have a negative influence on the development of the brain, with decrease of cell number, cell size, neuron dendritic length and arborization, synapse formation and amounts of various enzymes (Shoemaker and Bloom, 1977).

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In the brains of adult animals there is a correlation between the metabolic activity and the density or specific length of capillaries in various regions (Dunning and Wolff, 1937; Campbell, 1939; Horstmann, 1960). A rapid increase in the specific length of vessels is seen during the period of neocortical maturation, i.e., at the time of the neuronal cytodifferentiation and the development of aerobic glycolysis (Gyllensten, 1959a; Bär and Wolff, 1973). Since all nutrients are transported to the differentiating neocortex by the blood vessels, this increase in transport capacity is probably a prerequisite for the subsequent process of cellular differentiation. Consequently, a reduction of vascular growth might cause a nutritional strain on the developing cortex.

The rate of mitosis as well as of normal cell death is reduced in protein deficient animals even in organs other than the brain, e.g., the intestine and the liver (Deo and Ramalingaswami, 1965; Wiebecke et al., 1970; Leduc, 1949; Tongiani, 1971; Patel et al., 1973). A reduced "endoglial index" (number of endothelial + glial cells/number of neurons) has been reported in the somatosensory cortex of 10-day-old rats after prenatal and neonatal protein calorie restriction (Siassi and Siassi, 1973). However, the number of endothelial cells alone is not a sufficient basis for interpretation of the cause and functional consequences of reduced vascular growth in protein deficient rats. This paper reports a study of postnatal vascular growth in the cerebral cortex of protein deprived and normal rats in terms of specific length, specific surface, volume fraction and luminal diameters of vessels.

Material and Methods

Animals

Male rats of the Sprague-Dawley strain were examined at 7, 10, 12, 15, 17, 20, 30, and 90 days of age. Each age group consisted of six to

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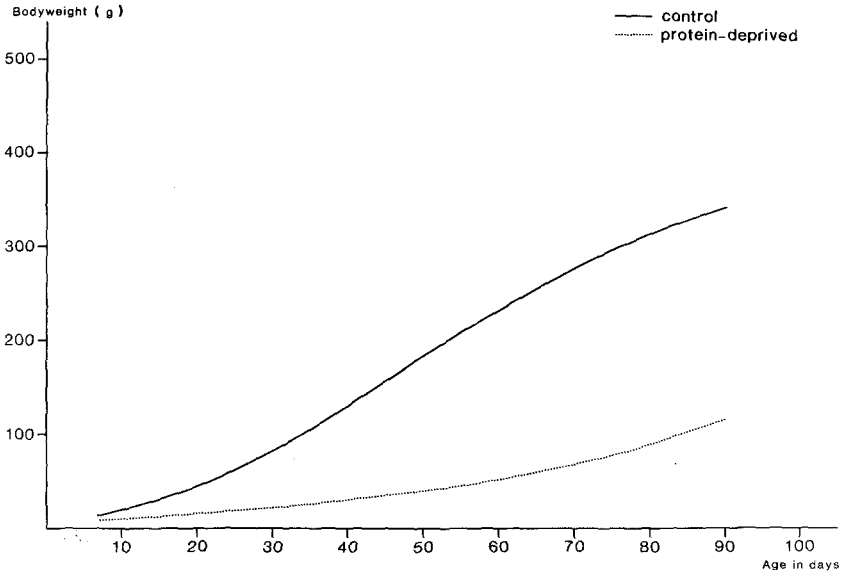

Fig. 1. Body weight of rats (g) during postnatal development (days)

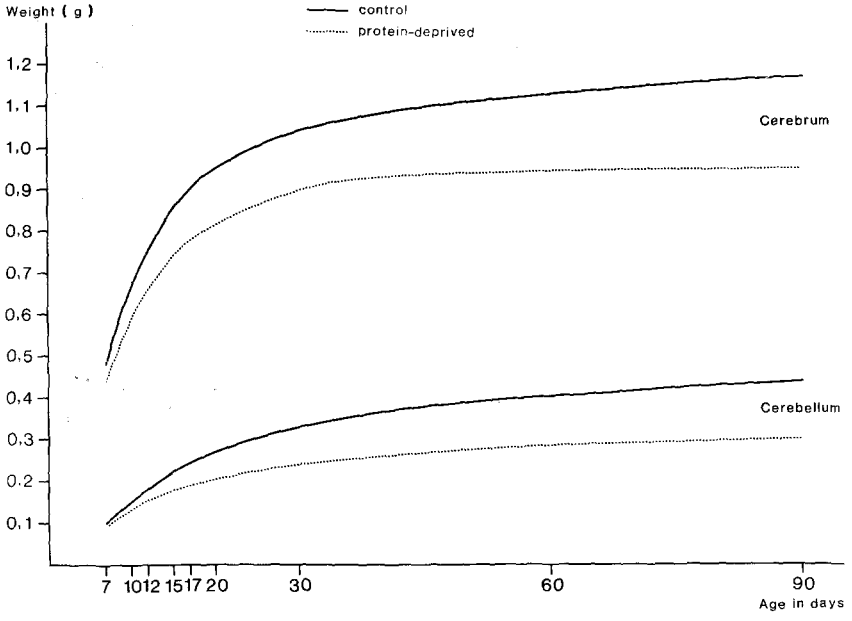

Fig. 2. Weight of the cerebrum and the cerebellum (g) during postnatal development (days)

eigth animals. Half the rats were subjected to protein deprivation. The protein content in the diet of control rats was 16 calorie %. Mothers of protein-deprived rats (PD) were kept on a diet containing only 8 calorie % protein, the other 8 being substituted for with starch, from 2 weeks before mating and throughout the suckling period. After weaning, the respective diets were given to the offspring. Water was given ad libitum. The amounts of vitamins and minerals were considered to be above the daily requirements in both groups. This procedure resulted in a marked decrease in body weight and gain in brain weight in the PD rats compared to the controls (Figs. 1 and 2). All rats were kept in plastic cages in a room with 60 % humidity and a temperature of 24° C. The room was illuminated from 6 a.m. to 6 p.m.

Experimental Procedure

Rats were perfused via an intracardiac needle with 50 – 600 cc of 3 % glutaraldehyde + 3 % paraformaldehyde in 0.1 M phosphate-buffered solution over 30 min. The brains were removed, weighed and postfixed for 4 h in the same solution. Specimens were cut out under a dissection microscope and rinsed overnight, dehydrated in graded ethanol series and embedded in JB-4 Embedding kit (Sorvall). The specimen area was measured on micrographs of specimens taken during preparation and a factor correcting for shrinkage due to preparation (K) was calculated from the specimen area after fixation divided by the area after dehydration (Eins and Wilhelms, 1976).

Sampling and Statistical Method

One 2 µ-thick section per animal was cut from a specimen of the left hemisphere area 18 (visual association, Krieg, 1946) and stained according to the method of Richardson et al. (1960). At a magnification of 60 or 80 ×, light microscopic images were scanned by a TV

of age and one from 20 – 90 days of age. During the first period there was a rapid increase in all three variables (Figs. 3 – 5).

Although small fluctuations in the rate of increase were present in the Lv from 7 – 20 days of age, we could not detect a significant difference from linearity. At 20 days of age the Lv reached 88 % of adult values.

The Lv appeared to be equally distributed through the cortex between 7 and 17 days of age but at 20 days of age a small peak had formed in layers 4 – 6, corresponding to the cortical lamina IV (Fig. 6). This peak of the Lv became successsively more marked and a new peak formed in layers 8 – 9, corresponding to lower V – upper VI, during the second period. The Sv and Vv showed the same phases as the Lv during development (Figs. 4 and 5).

Protein-deprived Rats

The vascular development in the neocortex of protein-deprived rats differed from that in the controls. At 7 days of age no difference in the Lv, Sv, or Vv was seen in the protein-deprived rats compared to the controls (Figs. 3 – 5). The Lv increased only slightly between 7 and 10 days of age in the protein-deprived rats, leading to a significant reduction compared to the controls. Between 10 and 20 days of age the Lv in the protein-deprived rats increased at approximately the same rate as in the controls but at 20 days of age the Lv of the protein deprived rats was only 60 % of the adult value, compared to 88 % in the controls. At 30 days of age the Lv was still reduced in the protein-deprived rats but at 90 days of age no significant difference could be demonstrated between the two nutritional groups.

As in the controls, the vessels appeared to be equally distributed throughout the cortex during early postnatal development. The formation of the peak in layer 4 – 6 was delayed since no distinct peak could be seen even in the 30-day-old protein-deprived rats. The cortical distribution of vessels did not differ markedly between the two nutritional groups at 90 days of age.

The Sv and Vv of the protein-deprived rats deviated only slightly from controls during development. However, there appeared to be a tendency towards somewhat lower values in the Sv during early postnatal development. There was also a marked reduction in the Lv of the thinner vessels ($\varnothing < 8.25\,\mu$). whereas the wider vessels ($\varnothing > 8.25\,\mu$) showed no reduction during development (Fig. 7). The mean luminal diameter of capillaries was higher in the protein-deprived rats than in the controls up to 30 days of age (Fig. 8).

Discussion

In the adult brain a correlation between synaptic density and metabolic activity on the one hand and capillary density on the other has been described (Dunning and Wolff, 1937; Campbell, 1939; Horstmann, 1960). The formation of synapses on the soma or spines of the young Purkinje cell initiates cytodifferentiation and outgrowth of the primary dendrite (Fox et al., 1967; Woodward et al., 1969; Altman, 1972; Kornguth and Scott, 1972).

As a results of the formation of synapses on the soma or dendrites of neurons, the number of mitochondria and the level of oxidative enzymes, such as succinic dehydrogenase, increases (Altman, 1972; Palay and

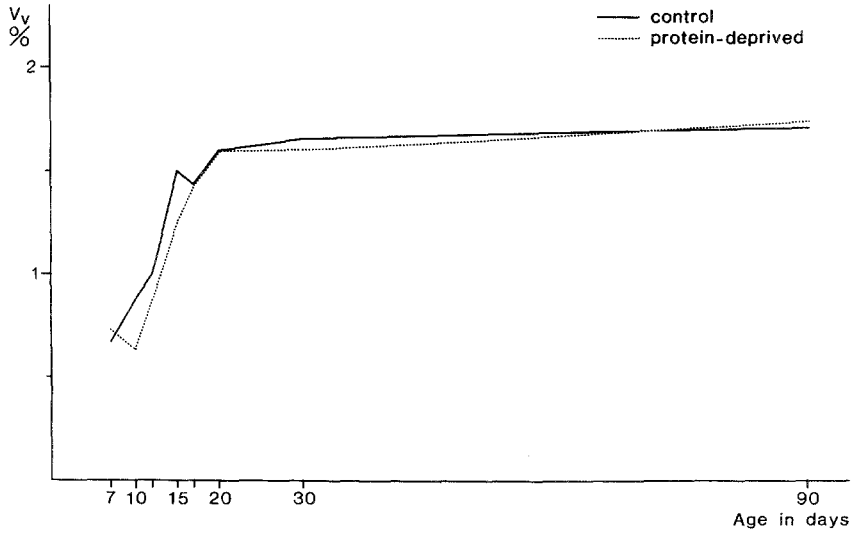

Fig. 5. The volume fraction of vessels Vv (%) in rat neocortical area 18 during postnatal development (days)

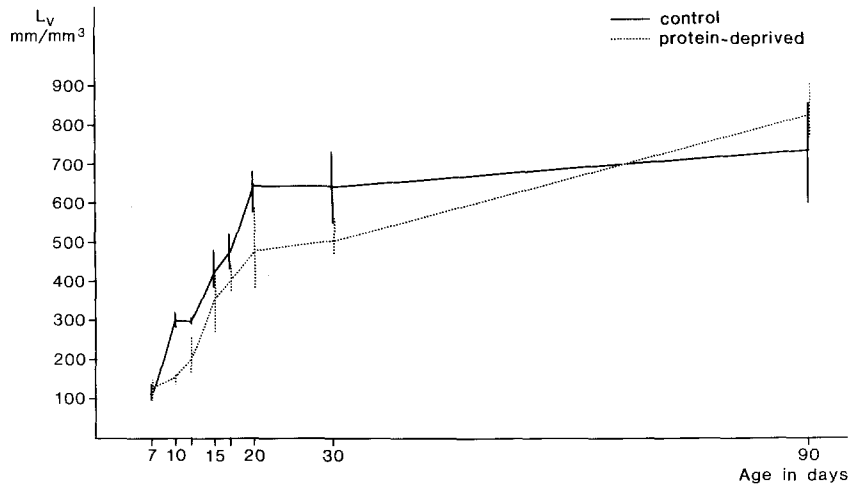

Fig. 3. The specific length of vessels Lv (mm/mm^3) in rat neocortical area 18 during postnatal development (days). Vertical lines represent total interindividual variation

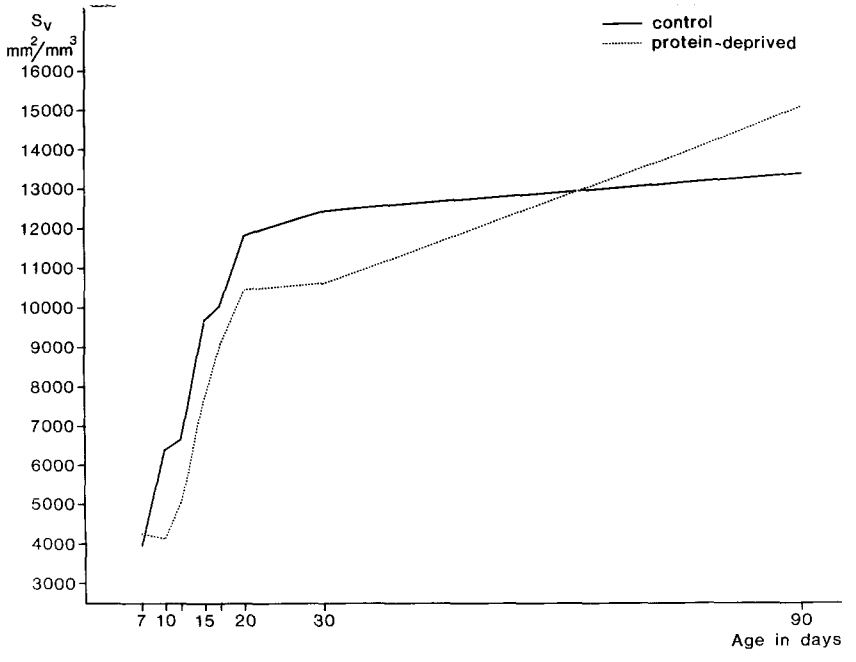

Fig. 4. The specific surface of vessels Sv (mm^2/mm^3) in rat neocortical area 18 during postnatal development (days)

camera. The sampling procedure used was systematic, four or more consecutive rows of ten consecutive field areas through the cortex being examined. Areas with large penetrating vessels were avoided since the capillary density is low in the vicinity of these vessels (Saunders et al., 1971). Vascular variables were measured by an automatic TV-analyzer (Quantimet 720, Metals Research, Cambridge) using the contrast differences between the empty vascular lumina and the stained surrounding tissue. The specific length of vessels (Lv), the specific surface of vessels (Sv), and the volume fraction of vessels (Vv) was calculated from the number, the total circumference and the area of vessel lumina per field area, respectively. For vascular (i.e., luminal) diameters the small axis of cutting figures elipses parallel to one of the sides of a cirumscribed rectangular frame rotated in steps of 45° were included. Theoretically, the area proportion of the lumen and the rectangle of accepted vessels approaches that of a circle and a square " $\pi/4$". Capillaries were defined as vessels with a luminal diameter smaller than 8.25 µ.

Vessel were also controlled for random orientation in tissue by comparison of the observed distribution for axial ratios of the cutting elipse with those theoretically expected for randomly oriented cylinders (Eins and Bär, 1978). The section thickness was not included in the calculations because data correction would not have improved the results since the section thickness was favorable. The shrinkage, on the other hand, could be compensated for by Lv/K (dimension $1/mm^2$), by $Sv/\sqrt{K}$ (dimension 1/mm) without compensation for the Vv (dimensionless). Differences between the PD and the control rats were tested with Fisher's permutation test and regarded as significant when $P < 0.05$.

Results

Control Rats

The neocortical vascular development of the control rats, as illustrated by the Lv, Sv, and Vv, could be roughly divided into two periods, one from 7 – 20 days

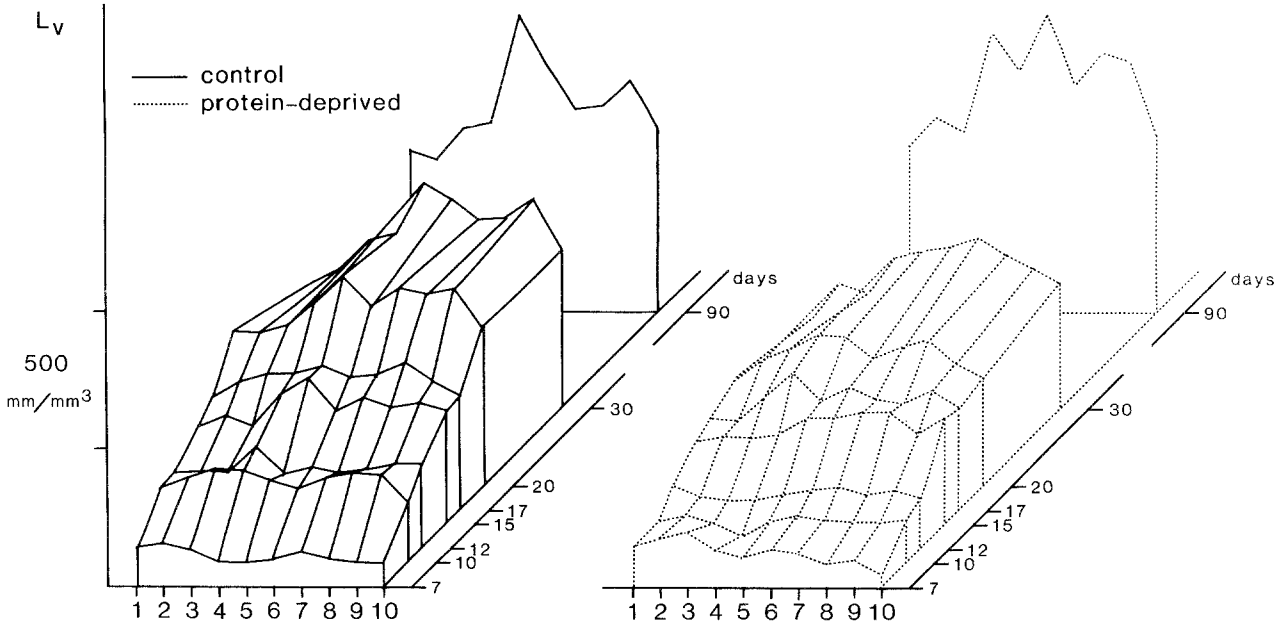

Fig.6. Cortical distribution (1 – 10) of the specific length of vessels Lv (mm/mm^3) during postnatal development (days). One (1) to 10 represents one tenth of the cortical thickness, 1 being the most superficial part

Chan-Palay, 1974; Woolsley and van der Loos, 1970; Farkas-Bargeton and Diebler, 1978). In the neocortex these maturational processes and the subsequent volume growth start in lamina I and proceed to VI, V and successively more superficial layers (Marin-Padilla, 1970; Fox et al., 1966; Conel, 1959; Friede, 1959; Farkas-Bargeton and Diebler, 1978). Recent results indicate that the maturation of the neocortex is related to the appearance of early maturating GABA-accumulating neurons (Wolff, 1978).

Earlier reports on the rapid postnatal vascular growth in the neocortex have emphasized the temporal link between its onset and the neuronal cytodifferentiation and development of aerobic glycolysis (Gyllensten, 1959a, 1959b; Bär and Wolff, 1973). However, since synapse formation appears to be a primary developmental step, it seems reasonable to regard the vascular growth as directly or indirectly linked to the synaptogenesis. A correlation between vascular and synaptic density of regions in the nervous system has also been demonstrated by Dunning and Wolff (1937). The number of synapses increases rapidly from the 8th postnatal day in the rat but no gross variations in the synaptic density are present between the different cortical laminae (Wolff, 1978). Since no laminar differences are present in the relative or specific length of vessels either (Craigie, 1938; own results), vascular length must increase at a rate proportional to the synapse formation as both increase equally throughout the cortex, irrespective of any differences in the volume growth between the six laminae during the postnatal period. The correlation between synaptogenesis and vascular development is further supported by recent findings on vascular growth in the cerebellar cortex (Conradi et al., 1979a, 1979b).

It has been proposed that the development of the previously mentioned GABA-accumulating neurons leads to the establishment of borders between cortical laminae as well as between the three sets of vascular modules that together feed the cortical capillary network (Wolff, 1978). A laminar pattern resembling that of the adult cortex is formed in vitro in cortex specimens isolated from newborn mice, i.e., independently of the afferent input (Seil et al., 1974). Gyllensten (1959b) has shown that if newborn mice are deprived of visual stimulation (darkness) the growth of the visual cortex is more affected than the vascular development in the same region, leading to increased relative vascularity.

In the central nervous system new vascular branches grow primarily as sprouts with non-patent lumina (Caley and Maxwell, 1970; Wechsler, 1965). The relative number of ramifications increases in the cortex before any increase in specific length of vessels can be seen (Bär and Wolff, 1973). Each sprout is initiated by endothelial cell mitosis but the branch increases its length more by an increase in its mean endothelial cell length than in its number of endothelial cells (Bär and Wolff, 1973).

It is clear from the results of our study on the normal cortical vascular development that the length of

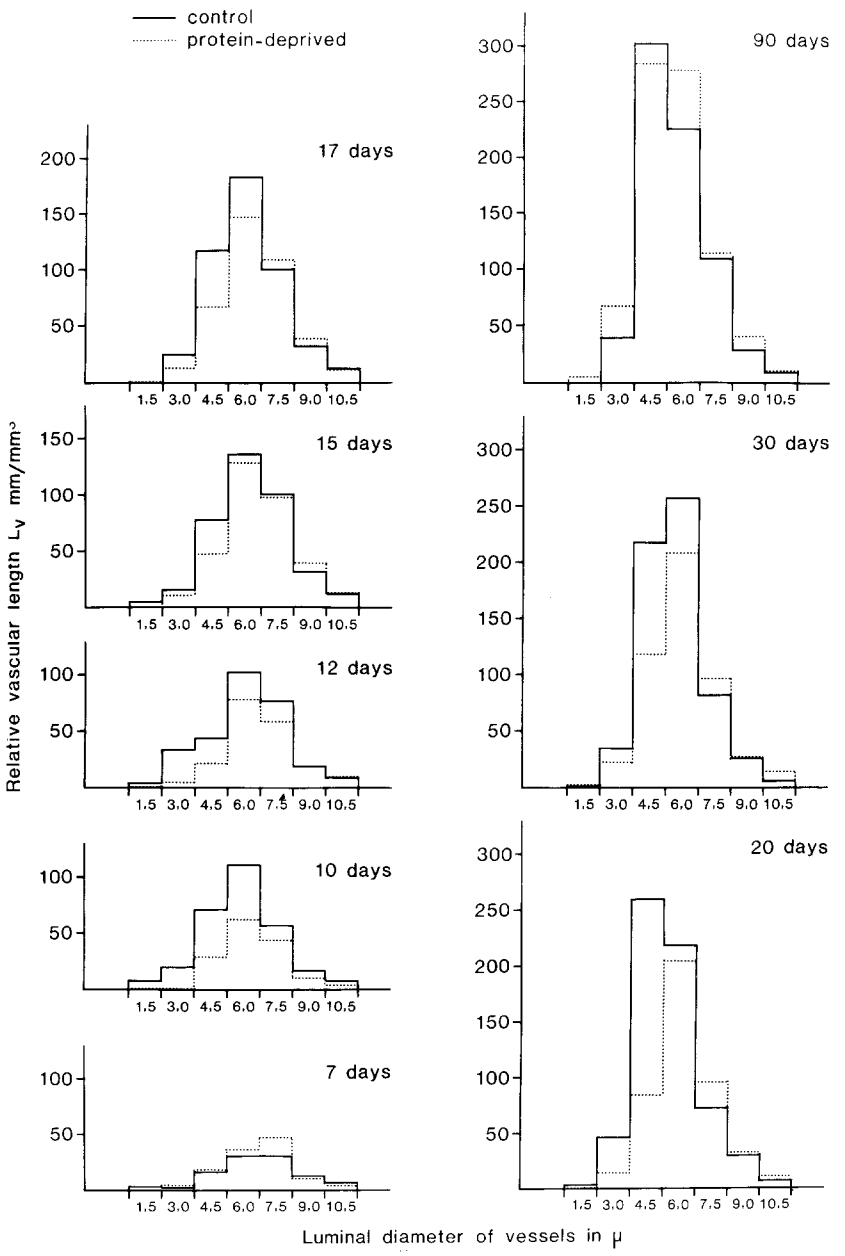

Fig. 7. Size distribution of luminal diameters expressed as specific length of vessels for each fraction at the ages examined (days). Figures on horizontal lines give the mid-point of each fraction in μ (0.75-[1.5]-2.25)

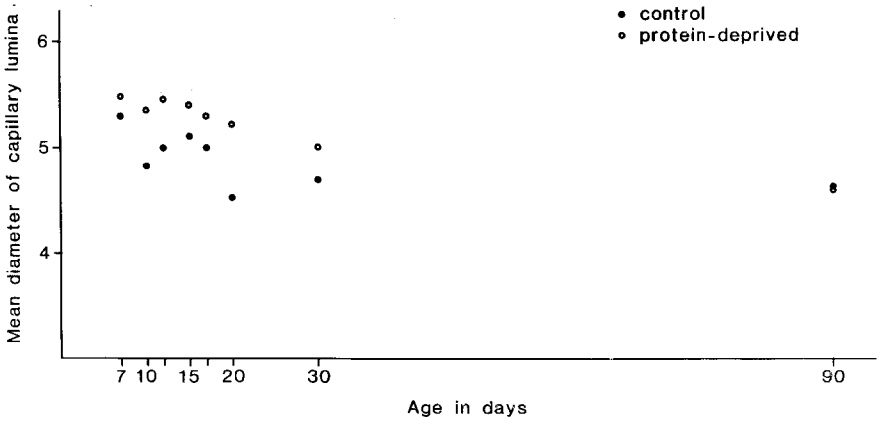

Fig. 8. Mean diameter of capillary lumina (μ) during postnatal development (days)

the wider vascular segments ($\varnothing > 8.25\,\mu$) increases during postnatal development. This presumably reflects gradual increase in the luminal diameter of vascular segments that is probably partly due to the formation of new branches with a consequent increased demand on the transport capacity in the pre-branch segments.

Two possible mechanisms may be considered when attempting to explain the decrease in vascular growth in the protein-deprived rats. Firstly, according to the previous reasoning, the decrease may be explained by a reduced synapse formation in the cortex of protein-deficient rats, as earlier reported by others (Cragg, 1972; Gambetti et al., 1974). In this context, it is also of interest to note that altered activities have been described for enzymes involved in GABA-metabolism in protein-calorie malnourished rats (Rajalakshmi et al., 1974; Katiyar et al., 1976). Secondly, the decrease in vascular growth in the protein-deprived rats may also be due to a decreased mitotic rate of endothelial cells, caused directly by the protein deprivation, as has been described for other cell populations (Leduc, 1949; Wiebecke et al., 1970; Deo and Ramalingaswami, 1965; Tongiani, 1971).

The reduction in the Lv of protein deprived rats was merely due to a decrease in the length of thin vessels. The mean luminal diameter in the capillary fraction ($\varnothing < 8.25\,\mu$) was larger in the protein-deprived rats than in the controls.

These findings may explain the differences between the Lv, Sv, and Vv of the protein-deprived rats compared to the controls. The increased relative length of the larger vessels in the protein-deprived rats compared to the controls should be considered in the light of a probable decrease in volume growth (Shoemaker and Bloom, 1977). However, the results indicate that the hypertrophic phase of endothelial cells was less influenced by the protein deprivation than the endothelial mitotic rate. The general tendency towards increased vascular diameters in the protein-deprived rats suggests an adaptation to the protein deficiency per se and/or to a secondary decrease in the formation of new vessels. Such an adaptation to lack of substrates has been suggested to occur in rats subjected to chronic hypoxia (Bär et al., 1975). In the present study the vessels were grouped only on the grounds of the luminal diameter and we have defined a capillary as a vessel with a luminal diameter smaller than $8.25\,\mu$, in accordance with other investigators (Bär and Wolff, 1973). It is possible that vessels which would be called capillaries on morphological or functional grounds are to be found in the $9.0\,\mu$ or even $10.5\,\mu$ fraction, especially in protein-deprived rats. If the terminal vascular branches are estimated to account for 90% of the Lv (Bär et al., 1975), a larger increase in the mean diameter of this fraction than in the "capillary" fraction would be present in the protein deprived rats compared to the controls. However, any compensatory increase in transport capacity must be regarded as functionally insufficient since the gain in brain weight was reduced in the protein-deprived rats compared to the controls.

Decreased density but increased luminal diameter of vessels in the cerebral cortex has been reported in rats made hypothyroid at birth (Eayrs, 1954). Although the underlying mechanisms of altered vascular development may be the same in cretinism and protein deficiency, it should be noted that the protein-deprived rats used in this study showed no obvious signs of hypothyroidism, such as edema or differences in the appearance of the fur (Beas et al., 1966). It is tempting to speculate that altered vascular development in protein deficient animals may lead to increased vulnerability of the brain to other pathogenic factors, e.g., hypoxia and hypoglycemia. Recent results indicate that mitochondrial succinate oxidation per unit brain is unaltered by prenatal protein deprivation (Muzzo et al., 1973). Heggeness (1962) found no difference in the survival time in hypoxia between postnatally protein-calorie malnourished and normal rats. This question deserves further evaluation.

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Note added in proof

According to present morphometric terminology the Lv should be read as "specific length of vessels". This term has been used in the legends and in the text. Unfortunately, the old term "relative vascular length" has been used in Fig. 7.