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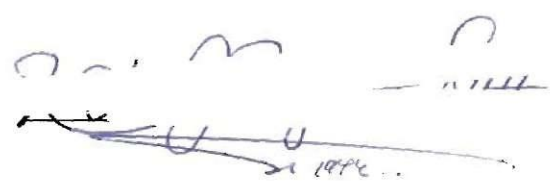

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HISTOPATHOLOGICAL AND CYTOCHEMICAL STUDIES OF FETAL AND NEONATE PRIMATE SPINAL CORD AFTER EXPERIMENTAL MATERNAL PROTEIN-CALORIE MALNUTRITION IN THE SQUIRREL MONKEY (*SAIMIRI SCIUREUS*)

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By

Neba J. Ngwa Suh

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HISTOPATHOLOGICAL AND CYTOCHEMICAL STUDIES OF FETAL AND NEONATE PRIMATE SPINAL CORD AFTER EXPERIMENTAL MATERNAL PROTEIN-CALORIE MALNUTRITION IN THE SQUIRREL MONKEY (SAIMIRI SCIUREUS)

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SUMMARY

This study was initiated to determine the histopathological and cytochemical effects of maternal protein-deficiency on fetal and neonate spinal cords of squirrel monkeys (Saimiri sciureus).

Female squirrel monkeys were arranged in a breeding colony with proven males, and watched over three estrus cycles. Pregnant monkeys were randomly distributed into three groups and fed Purina monkey chow 25 for about 45 days after conception during which time organogenesis was thought to be completed. Subsequently, two experimental groups were fed restricted specially prepared protein deficient diets of 4 percent and 8 percent content respectively while the control group had essentially the same diet except that the protein content was 25 percent, and all feeding was ad libitum.

A comprehensive examination in light microscopy of spinal cord sections during gestation and parturition for the effects of protein restriction was done. Cytochemical variations in enzyme activity on fresh and fixed frozen sections as well as histochemical changes in the morphology of paraffin embedded sections were studied.

The observations showed severe in situ changes in the morphologic and biochemical organization of spinal cord neurons during intra-uterine development of the experimental animals. There was pronounced chromatolysis of ribonucleoprotein complex in the neuroplasm while the nucleus showed reduced stainability with nuclear stains of hematoxylin-eosin, Feulgen and gallocyanin with RNAse digestion as control.

Many neurons exhibited disorganization within them while others had a general disorientation and condensation of chromatin material resulting in pycnotic, nuclei staining. An increase in the amounts of neuroglia surrounding chromatolysed neurons was noted.

The activities of several enzymes were examined and results showed variable distribution. Some activity apparently was disturbed (ATPase) or reduced (AC, IDPase and TPase) while other distribution appeared in increased (LDH, SDH and α-Gly-PDH) and some remained unchanged (AchE and AK).

The conclusion has been reached that protein-deficiency of pregnant mothers affects the fetal and neonatal squirrel monkey's spinal cord development. It would seem that extremely severe malnutrition results in an overwhelming distortion of the spinal cord and that such a distortion can not be resisted by the juvenile spinal cords. The possibility exists that protein deficiency at periods of active spinal cord growth and development (intrauterine maturation) may cause permanent deficits if it is severe and of long duration.

CHAPTER I

INTRODUCTION

Protein malnutrition during pregnancy in mammals represents a rather complex adaptation of both the maternal and fetal organs, not only to low protein intakes but also to altered tissue biochemical metabolism. The reduced protein levels in the maternal diet induce various pathophysiological and pathobiochemical alterations in the developing embryos. Intrauterine protein dificiency appears to adversely affect the central nervous system's protein synthesizing mechanism in addition to causing nerve cell number reduction and a decrease in size. Interference with the intracellular enzyme mechanisms may result in pathological changes within the neuron.

Subhuman primates provide a suitable model in which the pathogenesis of kwashiorkor may be suitably reproduced under defined dietary conditions, and the lesions induced carefully studied. Malnutrition in experimental animals may provide some relevant data that can be extrapolated to the human conditions resulting from similar malnutrition. Unlike other species, the developing subhuman primates have a relatively slow growth rate and a gradual development of a wide variety of behaviors. The processes and stages of development in subhuman primates are similar to those found in human development and show great resemblance to those of children (H. F. Harlow, 1959; M. K. Harlow and Harlow, 1966; Kerr, et. al., 1969).

The developing rhesus monkey, for example, does not attain a maximum level of intellectual development until sexual maturity while social behavior continues to develop and change from infancy through adolescence to adulthood.

The squirrel monkey, a subhuman primate, because of its size, availability, ease of housing and feeding in comparison to other larger primates provides an excellent model to study protein deficiencies during fetal central nervous system development. The simulation of conditions similar to those in human kwashiorkor in squirrel monkeys may help to delineate some aspects of protein deficiency. The squirrel monkey is a hardy animal and can accommodate protein malnutrition in a manner reflective of the human conditions. The squirrel monkey gestation period is approximately 167 days which means CNS growth rate is slower than other experimental species such as the rat with a gestation period of 21 days.

The present study was initiated to investigate the spinal cords of malnourished squirrel monkey fetuses and neonates after experimental maternal protein malnutrition. The specific purpose was to study in a coherent manner the histopathological and cytochemical alterations induced in spinal cords of fetal and neonatal offsprings whose maternal ad libitum diet intakes at pregnancy contained 4 percent and 8 percent protein-calorie while the control diet contained 25 percent protein-caloric content.

Whereas, 25 percent and 4 percent protein calories in the diet represent quite high and very low levels of protein, a group given

8 percent calories was introduced in order to see if they compensate for their deficiency by increasing the total food intake. Manocha (unpublished data) revealed that in the foods containing lower protein content, the total food intake is somewhat increased to compensate to some extent for the protein deficiency under the impact and demand of the growing fetus for more body building materials. Somewhat similar observations on the milk intake have been recorded in the growing rhesus infants by Kerr and Waisman (1970). They found that when the animals were fed the 1:1 dilution of the control diet (containing per liter: 25.4 gm fat, 70.5 gm lactose, 18.2 gm protein, and 5.2 gm minerals), they "immediately doubled their ad libitum intake, reaching about 300 percent of the normal volume intake per kilogram body weight during the first two months and between 300-400 percent of the normal volume intake during the rest of the deficiency phase of the experiment," which extended to two years of their age.

The study was considered significant because the parameters selected represented important metabolic signposts within the spinal cord cytoarchitecture, especially in relation to neuronal function. Hydrolytic enzymes catalize dephosphorylating activity through replacement by water of peptide ester bonds while oxidative enzymes function in the reactions that eventually liberate electrons from biochemical substrates. Acetylcholineesterase has intimate association with acetylcholine, the latter being involved with nerve impulse transmission. The question asked was what bearing dietary protein restriction had on the normal development and functional maturity of the cellular components.

CHAPTER II

REVIEW OF LITERATURE

Protein Malnutrition

Protein malnutrition remains an overwhelming and prevailing nutritional deficiency. The problem is identifiable not only in developing nations but has, in recent times, been associated with even the technologically advanced countries (Behar, 1968; Jelliffe, 1963). Protein deficiencies, as revealed by human and animal studies, have been linked with probable permanent changes in cognitive development and in the final level of intellectual growth (Brockman and Ricciutti, 1971; Cravioto and Robles, 1965). Estimates suggested that up to 70 percent of the preschool children in developing countries, are in all probability, suffering from some form of protein-calorie malnutrition (Coursin, 1965; Manocha, 1972).

Jelliffe, (1963) first designated protein-calorie malnutrition as a variable clinical syndrome, and later, the Joint Expert Committee on Nutrition of the FAO/WHO (1962) confirmed it. Described as kwashiorkor, protein malnutrition may refer to a wide range of physiological conditions whose clinical manifestations are associated primarily with children who have had extensive malnourishment. Edozien (1970) has indicated that protein deficiency, in its most severe form, is only identified in conjunction with other types of disorders. Mild forms of protein deficiency may be far more common

and probably less detectable although these may cause devastating damage to young children. The syndrome of protein malnutrition appears to develop as a result of imbalanced diet in the fetus and early childhood. The diet may be adequate in carbohydrates to supply calories but insufficient in protein content.

Despite variability in the manifestation of kwashiorkor syndromes, significant features have been established. Waybourne (1968) indicates irritability and resentfulness in moderate cases while apathy, misery and lethargy are significantly expressed in extreme pathologic states (Latham, 1968; Waybourne, 1968). Several investigators note retarded growth (Gopalan, 1968; Whitehead and Dean, 1964) and motor development (Gerber and Dean, 1957; Waterlow, Cravioto, and Stephen, 1960) in apparent kwashiorkor. Loss of appetite, reduced curiosity and inhibited explorative behavior, coupled with psychological disturbances are also constant manifestations of the syndrome (Clark, 1951; Trowell, et al., 1954; Cravioto, 1970). The child shows a lack of interest in his surroundings and has a very limited attention span (Burgess and Dean, 1971). In chronic cases, edema (Srikantia, 1968), brittle and sparse hair, (Jelliffe and Welbourn, 1963; Monckeberg, 1968; Trowell, Davis and Dean, 1954; Viteri et. al., 1964), light coloured skin (Waybourne, 1968) with flaky rash (Trowell, Davis and Dean, 1954; Jelliffe, 1955) may develop. A synergistic relationship exists between secondary infection and protein deficiency. Kwashiorkor may be further complicated by infestations and infections as severe anemia in hookworm infection, dehydration in

infective diarrhea and tuberculosis signs in the chest (Jelliffe and Welbourn, 1963).

Kwashiorkor may be regarded as a multiple etiology syndrome with a single pathogenesis. Lactating mothers, and children are more susceptible to protein deficiency because of the higher requirements for proteins needed as precursors in the synthesis of new body building blocks (WHO, 1965). The studies of Cabak and Vajdanvic (1965) showed that, despite apparent normal physical measurements, children malnourished in infancy and born of malnourished mothers, had inferior mental capacity. Children with histories of undernourishment show inability to integrate sensory information (Cravioto and Robles, 1965). Even if such children become rehabilitated, the results of some permanent alteration in mental development is indicated by inadequate performance as measured by the Gessell Infant Scale. (Gerber and Dean, 1957). Cravioto and Robles (1965) found that areas of motor development, adequate responses, language, personal and social development were not equally affected.

Malnutrition and Central Nervous System Development

Nutrition and the central nervous system development have been experimentally associated since the turn of the century. Donaldson, et al. carried out extensive investigations of the effects of malnutrition on the nervous system of adult and young rats (Jackson, 1925). Brain weights of weaned rats suffered very little from malnutrition (Hatai, 1908; Donaldson, 1911; Jackson, 1915). Stewart (1918) and Sugita (1918) independently observed that malnutrition

initiated between birth and weaning produced rat brains that weighed below normal for their corresponding body weights. Keys and coworkers (1950) investigated volunteer adult human subjects under conditions of severe malnutrition and concluded that mental performance levels were reduced in the subjects. The effect, however, was transitional in nature, and rehabilitation removed the transitory effects. The adult central nervous system was not subject to permanent stress resulting from malnutrition.

Experimental evidence has since accumulated to support these earlier findings (Barnes, 1967; Coursin, 1968; Dobbing, 1968, 1969, 1970; Miller, 1969; Platt and Stewart, 1969; Stewart, 1970; Winick, 1969,1970b,c). Malnutrition, it appears, may retard the process of central nervous system development, especially if it applied during the active phase of cell division. Dobbing (1968) indicated that in pigs and rats myelination was permanently reduced by malnutrition during brain development. He concluded that the brain, at its most active proliferative phase of growth, was very vulnerable to nutrition deficiency.

Most of the studies reviewed suggest that malnutrition, especially maternal, interferes with intrauterine growth and development of the fetus. Although it would seem that the fetus is protected by the maternal environment, the mother's condition of health and nutrition at pregnancy may adversely affect its development (Winick, 1968). The brain is especially vulnerable since most of it is formed prenatally. The studies of Winick and Noble

(1966) and Winick and Rosso (1969) show that total DNA content is reduced in children and rats subjected to early malnutrition. This can be explained in terms of the curtailment of cell division during the active cell proliferation stage of the central nervous system development. The peak period of human central nervous system growth is prenatal (Dobbings, 1969) and malnutrition initiated during pregnancy is more likely to yield permanent damage to the brain and spinal cord (Winick, 1969). The timing of malnutrition appears to be significant, whereas, adult brains may suffer transitory effects only (Keys, et al., 1959), the infant central nervous system is very vulnerable. Miller (1968) described the brain as solely responsible for perception, learning, problem solving and regulating a number of vital physiological and endocrinological functions in the body. Any permanent damage to the central nervous system would adversely affect its capacity to perform these functions. Although the adult brain appears to be "spared" when compared to other organs in nutritional deficiencies, the fetal brain and spinal cord would seem to be adversely affected during gestation. The functional impairments that are likely to result, may produce more serious consequences in later life. In addition, the dynamic equilibrium existing within the cellular components of the brain is also upset such that the RNA and protein synthesizing mechanisms become disoriented. Winick, et al., (1969) further indicate that the cell number and individual cell size is reduced permanently if malnutrition is initiated during intra-uterine growth.

The impairment that results from metabolic inadequacies of maternal food supply, especially in the available protein precursors within the growing fetus, may later be reflected in retarded mental performances (Cravioto, et al., 1969; Cravioto, 1970). Malnutrition of the pregnant mother can have devastating consequences in the offspring in later life.

Morphological alterations occur in various organs including the brain and spinal cord of animals after protein malnutrition (Dobbing, 1965; Dobbing and Widdowson, 1965; Winick and Noble, 1966; Benton, et al., 1966; Chase, et al., 1967). Cats (Ferraro and Roizin, 1942), pigs (Lui and Windle, 1950; Platt and Stewart, 1960; Lowery, et al., 1962; Platt, et al., 1965; Dickerson, et al., 1966; Stewart and Platt, 1968) and dogs (Platt and Stewart, 1969) seem to have similar structural effects from protein malnutrition. These alterations are more magnified if the animals are malnourished prenatally during their periods of rapid growth.

Pig brains, after one year of malnutrition, contain higher cholesterol levels but resemble brains of 10-12 week old piglets (Dickerson, et al., 1966). Spinal cords of malnourished pigs show marked morphological alterations in the anterior horn neurons (Stewart and Platt, 1968), while the brain tissue is edamatous and myelin sheaths are swollen. Platt and Stewart (1969) observed loss of Nissl substance from the motoneurons and concomitant higher numbers of neuroglial cells around such neurons in malnourished dogs. There is lost or reduced enzyme activity (glutamic and succinic dehydrogenases) and

curtailment of RNA content within the motoneurons of these malnourished dogs. It is inevitable that these derangements in neuronal structural may have functional significances.

The unique biological properties of the neuron, particularly regarding the dynamics of this specialized cell, indicate that concepts based on observations of the developmental processes of other cell types need not be valid for the nerve cell (Weiss, 1969). Ultraviolet spectrographic and volumetric studies have led to the conclusion that the embryonic mammalian nerve cells, unlike other somatic cells, pass through a second period of growth, characterized by continuous accumulation of nucleolar and cytoplasmic nucleotides and proteins (Hydén, 1943). Similar changes have been noted in embryos of chick anterior horn cells with regards to neuronal mass and nucleic acid content (Sourander, 1963; Hughes, 1955). Brattgard (1952) noticed in rabbit retinal ganglion cells differences of nucleoprotein and protein concentrations in young and adult or stimulated and unstimulated animals. Ringborg (1966) indicates similar changes in RNA content and in base composition of pyramidal cells of rat hippocampus.

Since Sugita (1917) reported the reduction in size of cortical neurons in rats undernourished from birth and Stewart, et al., (1969) have observed consistent abnormalities in spinal motoneurons of protein deficient pigs and dogs, it is pertinent that prenatal protein restrictions be examined in other species in order to determine if morphological and functional changes occur. Although, as pointed out

by Altman and Das (1966), rat macroneurons tend to form prenatally in contrast to glial cells which continue cell division postnatally, the very short gestation period and fast maturity of rats would preclude extrapolation of data obtained from rats to human studies. A similar conclusion may be reached with regards to pigs, dogs and other similar species used in malnutrition studies.

Protein Malnutrition and Subhuman Primates

The protein requirements of subhuman primates have not been determined accurately. However, Hodson, et. al. (1967) noted serum and urine changes of growing chimpanzes which suggest mild protein deficiency. De la Iglesia, et al. (1967) indicated that a soybean diet supplemented with methionine produced satisfactory growth of young squirrel monkeys. Day (1962) concluded from studies of young growing and adult rhesus monkeys that the protein requirements of the young were far greater than those of adult monkeys. On the other hand, Kerr, et al. (1969) noted that protein needs were reduced as month-old rhesus monkeys matured to one year of age.

The quality of protein has an immense effect on the utilization of dietary nitrogen (Allison, 1964; Munro, 1964). Anemia has resulted from interference with intestinal iron absorption in young rhesus monkeys fed a diet containing soy protein (Fitch, et al., 1964). Infant monkeys fed a diet in which maize provided the only source of protein developed apathy, hypoproteinemia and edema (Follis, 1957). Rhesus monkeys fed a diet deficient in phenylalanine demonstrated growth failure, hypoporteinemia, edema and anemia that were

corrected by biologically high quality protein or by the addition of phenylalanine to the diet (Kerr, et al., 1969).

The specific requirements of individual animals may depend on the total caloric intake, the quality of dietary protein, its digestability, the age and general health of the individual animal, feeding frequency and several other factors. The subhuman primate that demonstrates normal growth and normal physical, biochemical, and behavioral development is probably receiving adequate protein intakes. The excess intake of protein is not generally recognized as a significant health hazard except in states of hepatic or renal failure, therefore, in healthy individuals, excess intakes to a certain degree will probably not hurt (Gault, et al., 1968). However, it appears that food intake may be depressed and growth retarded when the diet is excessively high in protein content or contains imbalances of amino acids (Ashida and Harper, 1961; Sanahuja and Harper, 1962, 1963). Almquist (1954) believes that an accumulation of amino acids which can not be synthesized into proteins results in automatic impairments of appetite, thus reducing the ingestion of more amino acids.

Protein deficiency is a major health problem (McLaren, et al., 1970; Scrimshaw, et al., 1965) and many investigators have indicated that the sequallae of experimental malnutrition in growing subhuman primates are generally comparable to those in malnourished children (Follis, 1967; Kerr, et al., 1965, 1966. 1970; Mann, 1966, 1970; Racela, et al., 1966; Ramalingaswami, 1964, 1968; Sood, et al., 1965;

Waisman, et al., 1959; Ghitis, et al., 1963; Deo, et al., 1965; Wilgram, 1959; Wilgram, et al., 1958; Mann, et al., 1956). From all these studies, one may conclude that protein deficiency induces in primates lesions that closely mimic those found in human protein malnutrition.

Kerr, et al. (1970) studies of infant rhesus monkeys fed milk diets containing only 25 percent of the normal protein content but made isocaloric with lactose and fed ad libitum indicate growth failure, anemia, apathy, fatty liver metamorphosis, reduction in cardiac and skeletal muscle mass, and death. Surviving animals showed marked physical and behavioral "catch-up" growth after nutritional rehabilitation. Although malnutrition in children is rarely the result of a single deficiency of dietary protein, subhuman primates studies may be utilized advantageously to define the pathophysiology and biochemistry of amino acids as well as protein malnutrition in children at various stages of growth and development.

Subhuman Primates and Protein Requirements During Pregnancy

During pregnancy and lactation, metabolic rates rise and are accompanied by augmented needs for essential nutrients (Blaxter, 1964; Thomson, et al. 1970). Nitrogen, either in the form of intact protein or its constituent amino acids is needed in greater amounts for the synthesis of all structural or functional proteins. No definite data are available on the protein requirements for pregnancy and lactation in non-human primates; however, Portman (1970) has suggested that as in human requirements (Blaxter, 1964), the rhesus

monkey's needs increase about 25 percent in pregnancy and 50 percent for lactation. It is clearly difficult to ascertain to any measurable degree the protein needs occurring coincident to pregnancy. Though changes in the biochemical composition of the uterus, placenta, fetus and breast tissue indicate increased needs, many other changes occur in hormonal function, plasma binding proteins and metabolic activity and tend to complicate the picture.

Lehner, et al. (1967) have reported that 16 percent and 28 percent protein diets are indistinguishable with regards to the success of breeding squirrel monkeys, but Buss and Reed (1970) indicate that baboons fed diets of 7.2 percent protein during the later half of pregnancy and during lactation had less than normal breast milk while the growth of breast-fed infants baboons was substandard. Kerr (1968) studied the free amino acids in maternal and fetal blood at various stages of pregnancy and indicated that with the exception of taurine, lysine, 3-methyl-histidine and arginine, all free amino acids in maternal blood were lower at full term pregnancy than in nonpregnant adult females. Each free amino acid had a higher fetal blood level than that observed in maternal blood.

Harris (1970) has summarized in great detail the eating habits and nutrition of the laboratory animal while Portman (1971) noted the accumulated knowledge of the breeding and nutritional needs of the rhesus monkey. In contrast, the concise nutritional requirements of the squirrel monkey are not presently known but some investigative efforts have been made (Clarkson, et al., 1970;

Bulluck, et. al., 1969; Manning, et. al., 1969; Lehner, et. al., 1967; Manocha and Olkowski, 1972a,b; Olkowski and Manocha, 1972). These studies show that adult squirrel monkeys maintained on low protein diets show variable behavior. Animals become sluggish in movement, have sparse hair and indications of dermatosis are evident. Other changes include reduced chromatin and decreased RNA content of spinal cord motoneurons.

CHAPTER III

MATERIALS AND METHODS

Animals and Breeding

A colony of 40 healthy, sexually mature, female squirrel monkeys, maintained at Yerkes Regional Primate Research Center field station, was used for breeding purposes in outdoor facilities. Squirrel monkeys breed better in outdoors and good results have been achieved with this method (Clarkson, et. al. 1970). Animals were randomly assigned into groups of 11 or 12 females to one proven male over a breeding period lasting several estrus cycles. These animals were properly fed with Purina monkey chow 25 and well-cared for during breeding. The day of conception was ascertained by regular examination of vaginal smears as well as the presence of copulation plugs. Pregnancy was further ascertained by palpitation of the uterus. By regular examination and practice, the muscle tone of the uterus indicates the progression of the estrus cycle and based on the presence of sperms in the vagina, it is possible to pinpoint the day of conception with a fair degree of accuracy. Thirty-four females became pregnant over three estrus cycles of about 17 days each. After the animals were pregnant for about 30 days, they were transferred into an air-conditioned trailer which was monitored for constant temperature ($76^{o}F$), relative humidity and barometric pressure. The animals were randomly reassigned to a control

group of 12 and two experimental groups of 11 animals each. Each group was further separated into subunits of three or four per cage to allow for companionship yet avoid overcrowding. Each group was individually identified by a numbered neck tag. Throughout gestation, animals were observed at least twice daily in the mornings and afternoons for signs of abnormal behavior other than that induced by nutritional and other experimental stresses. The veterinarian regularly examined them for infections diseases. Individual animal body weights, food and water intakes were measured at specified intervals and their cages were regularly cleaned.

Diet and Feeding

The feeding regimen included an initial period of 45 days gestation in which all pregnant animals were fed Purina monkey chow 25. Presumably organogenesis should have been completed at this gestational age. The two experimental groups were then subsequently maintained on a protein-deficient diet of 8 percent and 4 percent casein respectively while the controls were maintained on a 25 percent protein feed till either Caesarean sections were performed or parturition returned.

The synthetic diet, as supplied by the manufacturer, was a pale yellowish amorphous powder. The dietary composition of the high protein food consisted of 25 percent casein, 61 percent corn starch, 8 percent vegetable oil, 2 percent Brewer's yeast, 4 percent salt mixture USP #2 (per 100 lb. feed: calcium biphosphate, 13.58 percent; calcium lactate, 32.70 percent; ferric citrate, 2.97 percent; magnesium sulfate, 13.70 percent; potassium phosphate, 23.98 percent; sodium biphosphate, 8.72

percent; and sodium chloride, 4.35 percent) and was fortified with vitamins (per 100 lb. feed: vitamin A, 200,000 units/g, 4.5 g; vitamin D, 400,000 units /g, 0.25 g; alphatocopherol, 5.0 g; ascorbic acid, 45.0 g; inositol, 5.0 g; choline chloride, 75.0 g; menadione, 2.25 g; p-aminobenzoic acid, 5.0 g; niacin, 4.5 g; riboflavin, 1.0 g; pyridoxine hydrochloride, 1.0 g; thiamine hydrochloride, 1.0 g, and calcium pentothenate, 3.0 g). The low protein diets had exactly the same quantified composition as the high protein one except that corn starch was substituted for the respective amounts of reduced casein. Care was taken to give the squirrel monkeys vitamine D_3 instead of D_2. The former is considered essential for their maintenance as well as breeding (Lehner, et al. 1967). Pellets were made from the powder by adding a fixed amount of corn syrup to measured quantities of the diet and its taste was augmented with an imitation banana flavoring. All groups of animals were fed twice daily at 9:00 a.m. and 4:00 p.m. The animals were given access to food and water ad libitum. The daily food ration was augmented with a small quantity of fruit salad containing variable combinations of lettuce, bananas, oranges and apples or peaches.

Fetuses and Neonates

Fetuses to be studied were obtained through Caesarean sections beginning with mothers whose pregnancy had lasted approximately 110 days (at least 65 days or more on control or experimental diet). The second batch was operated on at 140 days of pregnancy, while the neonate (newborn monkeys) specimens were available at term after a

gestational age of 167 ± 3 days (125 or more days of respective diet). Caesarean sections were carried out under general anaesthesia. The fetuses were removed and showed no ascertainable signs of being affected by the anaesthesia. Fetuses were immediately perfused with 10cc of physiologic saline solution (0.09 percent NaCl) through the umbilical cord followed by 20 cc of paraformaldehyde-glutaraldehyde fixative (4 percent paraformaldehyde and 0.5 percent glutaraldehyde). The dura mater was also slit open, and the exposed brain perfused with fixative. The entire perfusion procedure required speed and usually lasted no more than three to four minutes in duration. The whole fetus was then submerged in additional fixature for at least 24 hours. Spinal cords were subsequently dissected out of fetuses infused with minimum loss of time (two to three minutes approximately). The cords were cut into small sections and parts were immediately frozen in dry ice ($-78^{\circ}C$) and stored, after wrapping in aluminum foil, in stopped bottles which were frozen until needed for sectioning. The other parts of the cord were kept in buffered 10 percent formalin, subsequently processed and embedded in parafin. The neonates were treated similarly except that the spinal cord was also removed prior to fixation and frozen at dry ice temperatures. Sections were processed in the manner already described for the fetal cord. Histo- and cytochemical studies were performed on fresh frozen 10 µm-thick cord sections cut at $-20^{\circ}C$ in a Harris Cryostat then mounted on glass cover slips. Morphologic and histopathologic investigations were carried out on 4-6 µm-thick paraffin embedded tissues cut with a

Spencer "820" microtome and mounted in egg albumen on regular slides. Sections were studied in light microscopy and pictures taken with a Zeiss photomicroscope located in the Neurohistochemistry Laboratory, Yerkes Regional Primate Research Center.

Histological Methods

The sections were processed according to a standard histological procedure, (Barka and Anderson, 1963; Luna, 1968; Pearse, 1961; Lillie, 1965). The following staining methods were employed on paraffin sections belonging to fetuses and neonates from different animals.

Hematoxylin and Eosin (H & E), (Luna, 1968)

Alum hematoxylin and Eosin Y is the method preferred for a reasonable differentiation of nuclei and cytoplasm. Eosin is used as a counter stain for the contrasting effect when hematoxylin is used as the primary stain. Therefore, its proper differentiation by alcohol is very important in producing an H & E stain of high quality. Differentiation of both stains is equally significant and pertinent to the quality of stainibility.

Periodic Acid Schiff (PAS) and PAS-Diastase (PAS-D), (McManus, 1946)

The periodic acid-Schiff reaction is basic to carbohydrate histochemistry, being an adaptation of periodic acid oxidation introduced in chemistry by Malaprade (1928). The Schiff's reagent demonstrates aldehyde groups created by periodic acid oxidation in tissues. The PAS reaction evolved as a modification of Malaprade's method by a number of workers (McManus, 1946, 1948; Lillie, 1947, 1949, 1952 and Gomori, 1952). Glycogen is digested by diastase (0.8 percent

diastase for one hour at 37°C). Diastase treated and untreated sections are stained together. If PAS staining is abolished by the enzyme treatment, it indicates the presence of glycogen and no evidence exists that glycogen normally resists such digestion.

Einarson's Gallocyonin with RNase Digestion (Einarson, 1932)

Gallocyanin is an oxazine dye and acts in aqueous solution as a weekly acid stain. The gallocyanin-chrome alum procedure does not distinguish between DNA and RNA. Brachet (1940) introduced the use of RNase to digest RNA before staining as a control procedure. This eliminates any significant staining due to the presence of RNA, although the enzyme may not completely digest RNA from fixed tissues.

The enzyme is thermostabile in acid medium up to certain temperatures, and degrades ribonucleotides in two steps. Initially, a phosphodiesterase digestion results in pyrimidine nucleotide-2:3 hydrogen phosphate end groups. A secondary step involves hydrolyses of these groups to nucleoside-3 hydrogen phosphates which are readily diffusible. The definitive test for histochemical identification of ribonucleic acid is therefore its digestion by the enzyme ribonuclease. Lillie (1965) pointed out that long aqueous formalin fixations make tigroid substances (Nissl) and ribonucleic acid generally more resistant while brief alcoholic formalin fixation facilitates this ribonuclease activity of digestion.

Feulgen Reaction (Feulgen and Rosenbeck, 1924)

The definitive method for detection of DNA is the Feulgen reaction. It is specifically a DNA test that is dependent on gentle

and mild acid hydrolysis (1N HCl for 8 to 12 minutes at 60°C) of fixed tissues to form aldehydes. Subsequent application of Schiff's reagent (leucofuschin-sulphurous acid) to these exposed aldehydes yields a deep red purple (magenta) color complex in the nuclear chromatin. It seems that the Feulgen reaction occurs only with DNA (nuclear chromatin) but not RNA (nucleolus) or the cytoplasmic chromidial substance (RNA).

Masson's Trichrome(Masson,1929)

The effectiveness of this technique depends on the successful selectivity and specific uptake of molybodic and tungstic acids by the fibers and subsequent binding of the sulfonated amino triphenyl-methane dyes by their NH_2 groups (Puchtler, 1958). The acid and fibres stains are used sequentially while a mordant Bouin's fixative enhances staining of cytoplasm at the expense of other tissues (e. g., connective tissue) which renders them more difficult to stain fully. The trichrome method stains fibres of neuroglia, fibroglia nuclei, mitochondria and axis cylinders (axons). The nuclei are stained black while collagen red and mucus are black.

Enzyme Histochemical Methods

Standard histo- and cytochemical techniques were used to process sections and study enzyme activity (Manocha and Shantha, 1970; Pearse, 1961; Burstone, 1962; Barka and Anderson, 1963; Adams, 1965). The following histochemical methods were employed on fresh frozen and fixed frozen sections. A list of enzymes studied and methods used is summarized in Table 1.

Acid Phosphatase (AC)

Two procedures were used to demonstrate AC activity. The first procedure was the simultaneous coupling azo dye method as outlined by Barka and Anderson (1963) and Gregg and Pearse (1952). The second technique was the simultaneous coupling azo dye procedure used by Barka (1960).

Adenosine Triphosphatase (ATPase)

The lead method of Wachstein and Meisel (1957) was used to demonstrate the ATPase activity. This procedure is a modification of Gomori's lead nitrate method.

Alkaline Phosphatase (AK)

Two techniques were employed to show AK activity. The alpha-naphthyl phosphate method as used by Gomori (1951, 1952) was used first. A second method was the naphthol AS-BI phosphate azo dye technique as outlined by Burstone (1958, 1962).

Inosine Diphosphatase (IDPase)

The technique as outlined by Novikoff and Goldfischer (1961) was used. IDPase activity was demonstrated with sodium inosine diphosphate as a substrate.

Thiamine Pyrophosphatase (TPPase)

The activity of TPPase was demonstrated with thiamine pyrophosphate as the substrate. The method of Novikoff and Goldfischer (1961) was employed for TPPase.

Acetylcholinesterase (AchE)

The activity of AchE was investigated with two procedures. The

first technique was the direct coloring thiocholine method as used by Karnovsky and Roots (1964). The other procedure was the modified Koelle and Friedenwald (1949) method as outlined by Coupland and Holmes (1957).

Alpha-Glycerophosphate Dehydrogenase (α-Gly-DH)

The enzyme was demonstrated by the Nitro BT method as outlined by Hess, et. al., (1958). The substrate used was sodium DL-α-glycerophosphate.

Lactic Dehydrogenase (LDH)

The activity of LDH was demonstrated by the Nitro BT method used by Hess, et. al., (1958). Sodium lactate served as the substrate.

Succinic Dehydrogenase (SDH)

The Nitro BT method of Nachlas, et. al., (1957) was used to demonstrate SDH activity. The substrate used was sodium succinate.

CHAPTER IV

OBSERVATION

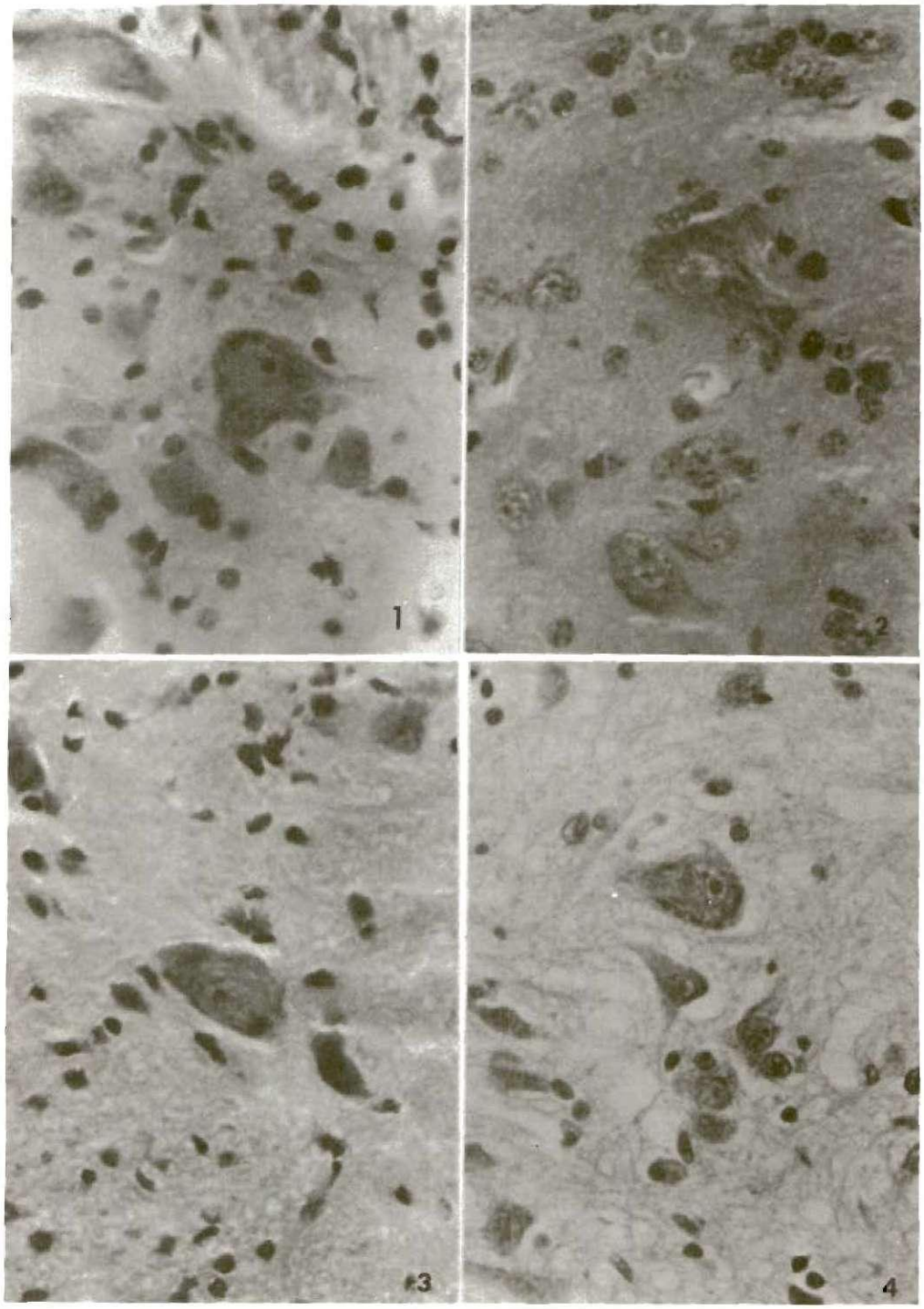
Morphological and Histochemical Studies

Morphological and histochemical observations were made on fixed spinal cords sections of both healthy and protein deficient fetal and neonatal squirrel monkeys.

Hematoxylin and Eosin Reaction

Spinal cord preparations from control animals show neurons that contain many uniformly spread chromatin granules in the nucleus. The prominent nucleolus is intensely stained and is centrally located within the nucleus. The neuroplasm has discrete, variably stained patches of flaky chromatin throughout with a greater concentration along the cell membrane. Some eosinophilic granules are visible in the proximal parts of dendrites. A few oligodendroglia show intense basophilia and are noted in the vicinity of the neurons. Ventral horn cells have stronger hematoxylin-eosin reactions than do the dorsal horn cells. Ependymal cells are characterized by strong basophilia as well as nuclei distributed near the cell base (Figures 1 and 3).

The nucleus of experimental animals shows a distinct network of crisscrossing strands of chromatin that radiates from the nuclear membrane to anchor the deeply stained nucleolus in either a central or an eccentric position. The neuroplasm contains eosinophilic material



Figures 1-4. Photomicrographs of Spinal Cord Neurons. Harris Hematoxylin and Eosin Stain (see p. vi).

that varies in staining intensity from a faint through moderate to strong reaction. The granules are dispersed in no definite pattern in the majority of nerve cells. Oligodendrocytes show deep basophilic nuclei and many of these cells are located within the vicinity of neurons. Some oligodendroglia are either in direct contact with the nerve cell membranes or are spread in rows and bunches along nerve processes (Figure 2). There is some mild rather than negligible eosinophilic reaction within the neurophil (Figure 4).

In most neurons, with the exception of the very strong reaction in the nucleolus, the nucleoplasm stains very weak, and the amount of chromatin granules is decreased. Other motoneurons show a stain gradient between the cell membrane and the nuclear membrane. Eosinophilia develops in favour of either membrane areas where darker parchy flakes of Nissl substance are seen, however, this is seen only in a small number of motoneurons. The strands of nucleoplasmic material that anastomose the nucleolus to the nuclear membrane diminish in number and intensity. Cell outlines appear fuzzy or blurred, ragged and become generally indistinguishable from the surrounding neuropil. The neuropil stains moderately and more cell processes become visible within the neuropil. A considerable number of neurons has attained the "ghost cell" stage with just remnants of Nissl substance stained while only the bare outlines of the nucleus show. The loss of chromatin from the nucleus is evident where only faint or opaque staining occurs.

There is a preponderant increase in neuroglia, particularly

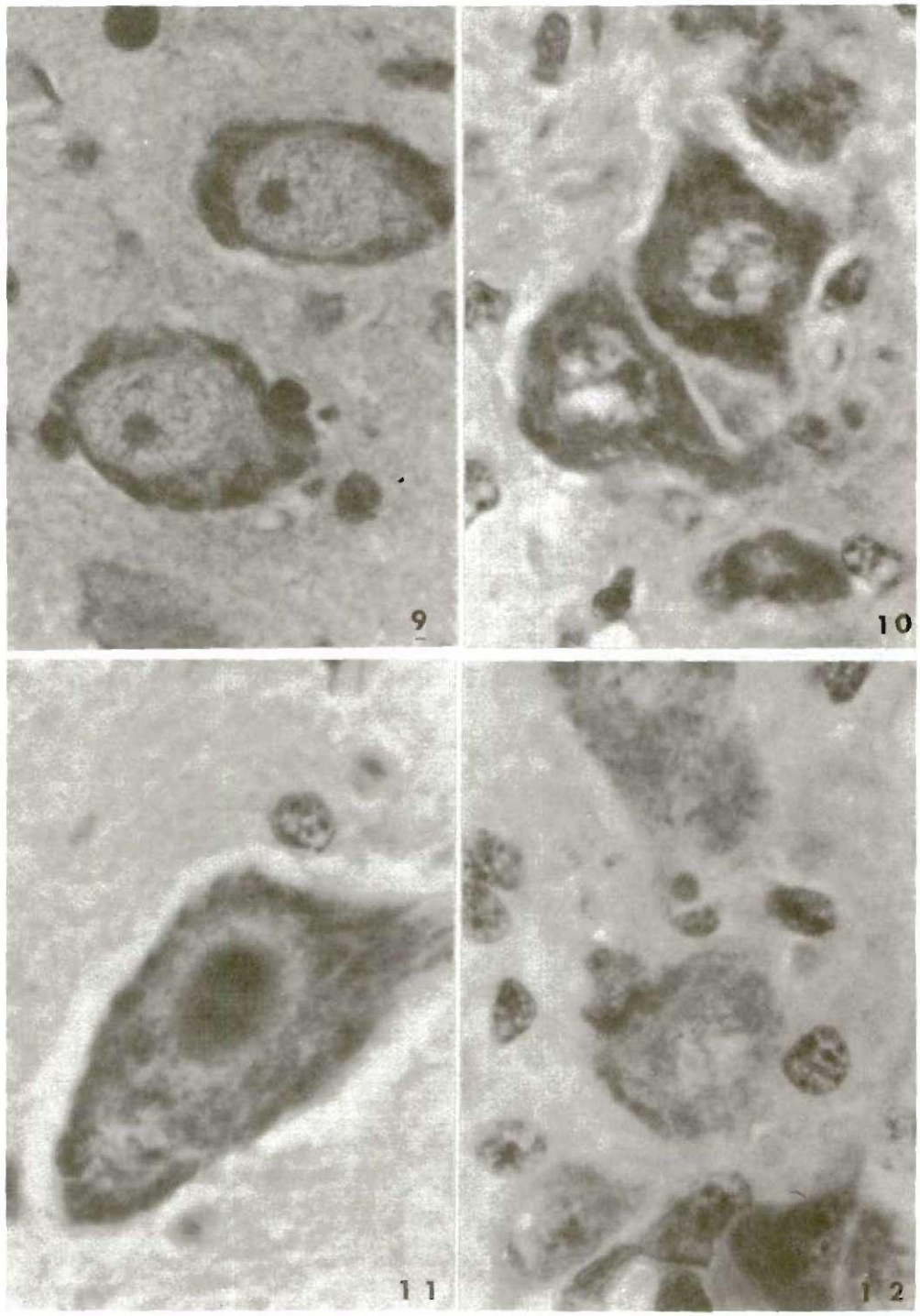
those in contact or only a short distance away from nerve cell bodies. Both the gray and white matter show immense increase in numbers of glial cells. Their nuclei are usually strongly stained. Other nerve cells have rather few glial cells around them or along their processes. The ependymal cells are mildly chromatolysed.

In contrast to the clear and distinct cell features of healthy spinal cord neurons, the malnourished neurons show significant vacuolation in the cell cytoplasm (Figure 4).

Periodic Acid Schiff Reaction

In control animal cells, PAS-positive granules are uniformly distributed within the neuronal cytoplasm. Some granules are densely clustered around the nuclear membrane. A majority of the granules are digested by the enzyme diastase, indicating the presence of glycogen (Figure 12). It is noted however that only partial digestion occurs within the cells and especially since hematoxylin was used as a nuclear counter stain. The glial cells have a small amount of PAS-positive granules, which disappear after digestion with diastase. The neuropil and blood vessels show mild positive reaction to the stain (Figure 9 and 11).

In comparison to control sections, malnourished animals have a greater amount of PAS-positive granules, especially within the neuroplasm where the reaction is very intense. The granules range from many large positive ones to few small-size types that are interspersed within the former. Diastase digestion is only partially successful in those neurons that have strong positive reactions.

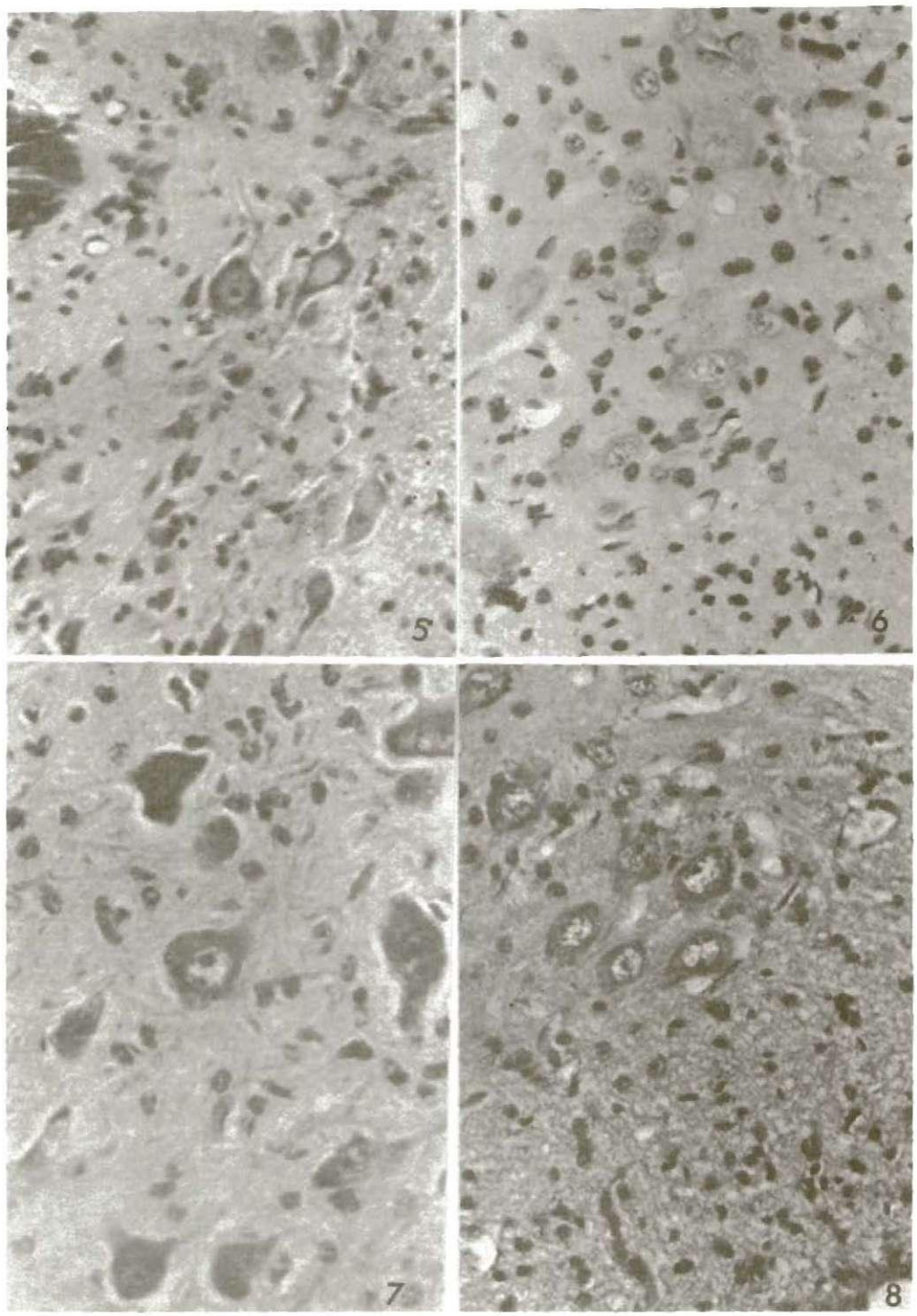


Figures 9-12. Photomicrographs of Spinal Cord Neurons. PAS-PAS-D Stain (see p. vi).

Blood vessels and the cell processes show moderate PAS-positive reactions. In neurons where large clusters of positive glycogen exist, the majority appear to be in the area just perinuclear while in others it is in the area just within the cell membrane. Other neurons show a strong positive activity throughout the cell. In the severely malnourished animals, the cell processes are very densely stained as are the glial cells. Diastase digestion is least effective in this group when compared to the control animals. The neuropil and pia mater are stained but not as intensely as are most of the other structures (Figure 10).

Gallocyanin Reaction

Control animals show gallocyanin-positive material about the cell nuclei, oligodendroglial nuclei and mild activity in the neuropil. A variable intensity is seen in the distribution of the granular substance. The ventral horn cells, because of their large size, show the strongest chromalum staining. The nucleolus is very positively stained while the nucleus is moderate in contrast. The neuroplasm also shows moderate chromophilic intensity, particularly just within the perinuclear area. The chromatin substance is not intensely stained in the neuroplasm proper but along the inner cell membrane surface concentrated chromalum-positive material can be seen. The few oligodendroglia within the vicinity of most of the neurons show strong basophilic nuclei while other similarly stained neuroglial cells are located in the white matter. The ependymal cells of the central canal show very strong gallocyanin staining (Figure 5).



Figures 5-8. Photomicrographs of Spinal Cord Neurons. Gallocyanin Stain (see p. vi).

Different stages of chromatolysis are observed in the neuronal cytoplasm of protein malnourished animal spinal cords. The nucleolus generally, is more eccentric and is located toward the nuclear membrane in its position within the nucleus. Thin strands of gallocyanin positive material radiate from the nucleolus to the chromatin granules located near the nuclear membrane (Figure 8). Some gallocyanin positive deposits cluster in small granular form on both the inner and outer surface area of the nuclear membrane. The karyoplasm is generally chromophobic. Oligodendroglia proliferate the nerve cell area and some come in close contact with the chromatolytic neurons. The neuroglia nuclei are strongly chromophilic (Figure 8).

Although the majority of severe protein deficient spinal cord sections represent an exaggerated state of the moderate deficiency, some unique features are observed in the neuron populations. Several neurons show dark, intensely stained neurons with the pycnotic nuclei also intensely stained (Figure 7). This may represent a condensation of chromatin material due to possible cell shrinkage or disorganization within the nerve cell. Other neurons have heterogeneously stained basophilic substance in the neuroplasm. The digestion of Nissl substance with RNAse removes all of basophilic material from the cytoplasm and part from the nucleus. This indicates that the material is RNA in nature (Figure 6).

Feulgen Reaction

There is very little distinction in the Feulgen reaction between

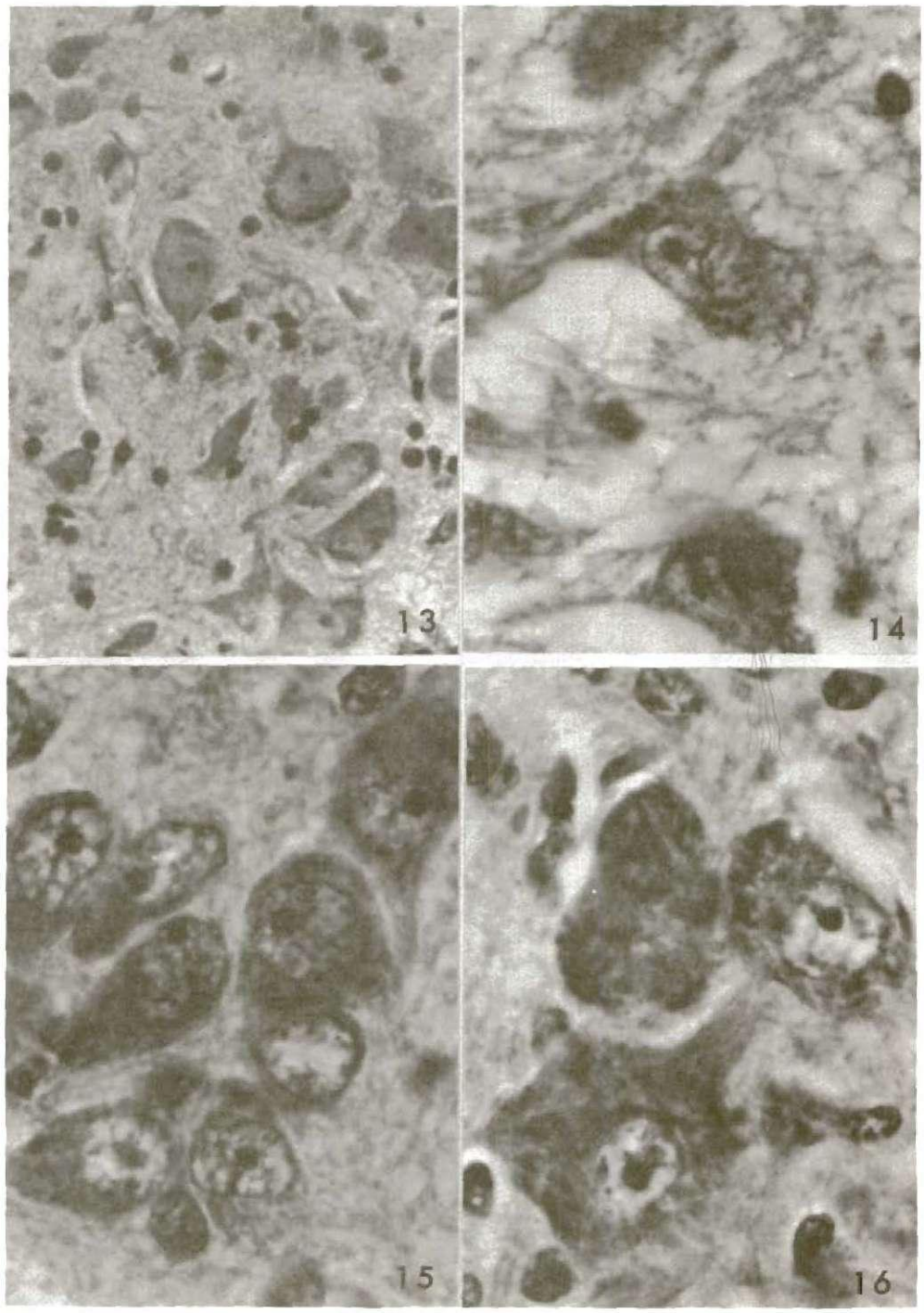
healthy and protein deficient animals. The control animals have well stained nuclei and it is difficult to distinguish the nucleolus from the rest of the nucleus. The distribution of Feulgen-positive material in the nuclei is generally irregular although some homogeneity may be seen in a few nuclei.

Experimental animals show within the nuclei of some neurons an apparent decrease in stainability as compared to the control group. However, conclusions about DNA content in these animals can only be speculative since no quantitative studies were made.

Masson's Trichrome Reaction

Masson's trichrome technique stains the neuroplasm rather uniformly and blue granules are evenly distributed in neurons of control animals. Prominent with the trichrome stains are the cell processes. These processes can be seen to radiate in all directions from the nerve cell bodies and within the white matter from the glial cells. Although, the nucleolus is stained a deep pinkish brown, the nucleus shows a pale more or less colorless structure. Blood vessels are well stained and can be seen in all areas of the cord sections. The neuropil stains red and may denote myelin, especially in the sheaths of axons (Figure 13).

Spinal cords of experimental animals have a variable trichromic reaction. Nerve and glial cell processes are well stained (Figure 15). Dendritic and glial processes can be seen originating from cell bodies in all directions and covering fairly long distances (Figure 16). The neuroplasm shows different staining characteristics to that of



Figures 13-16. Photomicrographs of Spinal Cord Neurons. Masson's Trichrome Stain (see p. vi).

control animals with a few exceptions. The decrease in number of basophilic granules in the cytoplasm is obvious and is in agreement with the previous observations on chromalytic neurons (Figure 14). Glial cells' proliferation in malnourished animals is significant and their nuclei are strongly stained.

Enzyme Cytochemical Studies

Cytochemical observations of enzyme activities included certain selected phosphatases, esterases and dehydrogenases. Significant amounts of tissue were perfused with fixative before the cytochemical evaluation was initiated. Fixatives adversely affect oxidative and to a lesser extent hydrolytic enzyme activity. Therefore, only preliminary results are given and no conclusions are drawn from the comparison between enzyme activity in experimental and control animals. Furthermore, since no distinctive differences in the reaction of the spinal cord to protein deficiency were indicated within particular experimental groups, all observations described represented data for all animals studied within the group. In addition, there were, obviously, some developmental differences between the particular groups studied as far as morphological changes are concerned. These were evaluated in terms of their severity and variation from control groups in relationship to the duration of protein deficiency. Table 2 represents results of enzyme activity studies.

Phosphatases

Acid phosphatase activity is strong in the cytoplasm of

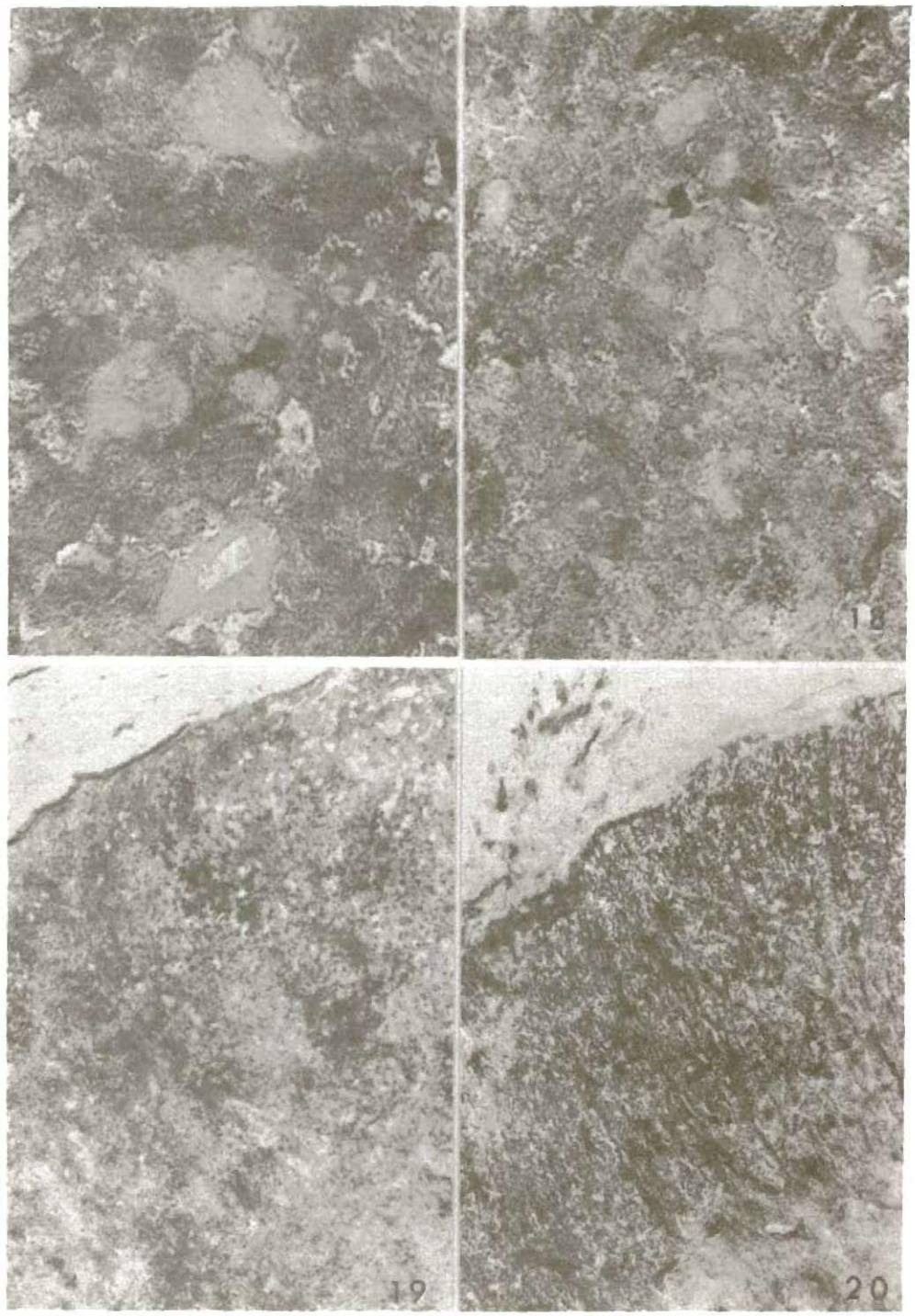
neurons particularly that of large ventral horn neurons of control animals. The neurons in the dorsal horns show from moderate to strong reactions. The main repositories of this enzyme are lysosomes which are evenly distributed in the perikaryon and the proximal parts of the dendrites of motoneurons in healthy animals (Figure 27).

Malnourished animals show a pattern of acid phosphatase that is not significantly altered from that of healthy monkeys. The neurons show moderate to strong activity while glial cells and neuropil show negligible to mild activity (Figure 28).

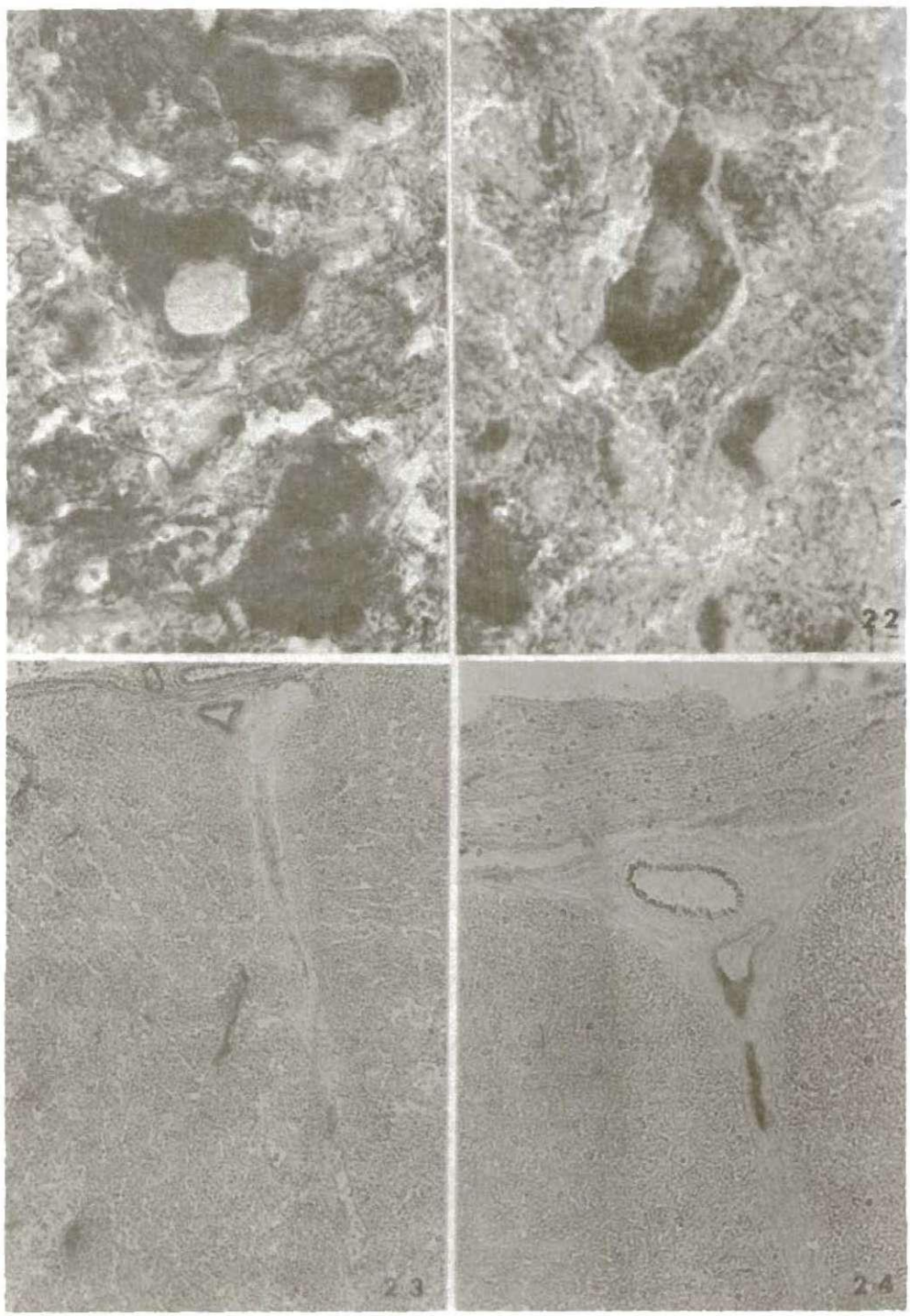
Alkaline phosphatase activity is very similar in all animals (Figure 23, 24). Capillaries are the mainstay of this enzyme's activity and the ventral fissure with its many blood vessels shows typical alkaline phosphatase reaction which range from mild to moderate.

Adenosine triphosphatase shows its strongest activity within the neuropil and the peripheral areas of the neuroplasm (Figure 17). Cell membranes have strong positive activity while the nucleus and nucleolus have negligible or negative reactions. The activity of ATPase in glial cells is very strong in the white mater in all animals although in the healthy animals the reaction appears more precisely localized (Figure 19).

Protein deficient animals show irregular distribution of ATPase activity and could be considered as reduced activity. Such a shift from the original sites may represent some functional adjustments or have significance in the total cell energy utilization (Figure 18, 20).

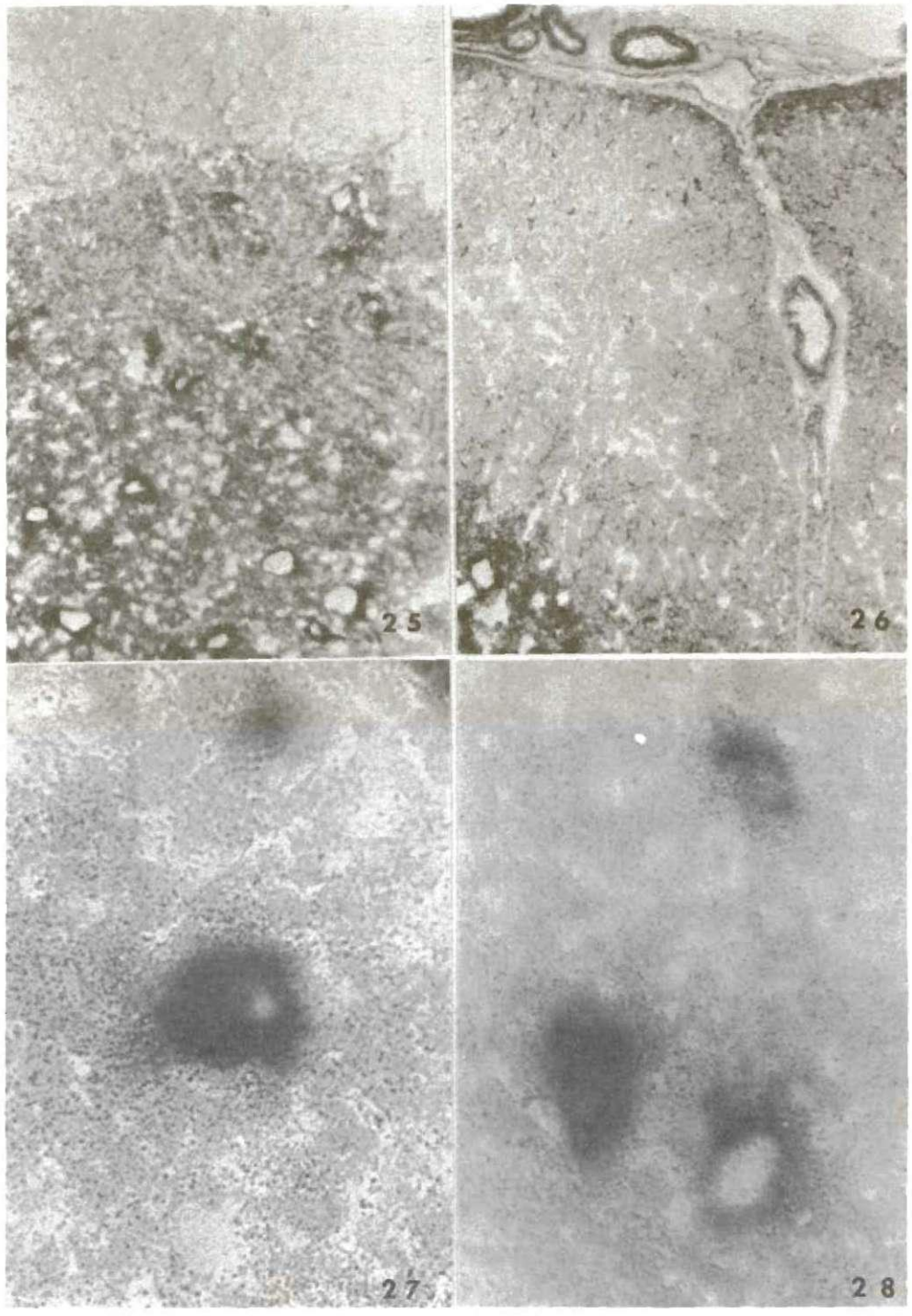


Figures 17-20. Photomicrographs of Spinal Cord Neurons. ATPase Activity (see p. vi).



Figures 21-22. Inosine Diphosphatase (IDH) Activity, (see p. vi).

Figures 23-24. AK Activity, (see p. vi).



Figures 25-26. LDH Activity, (see p. vi).

Figures 27-28. AC Activity, (see p. vi).

Inosine diphosphatase activity is strongest in the neurons especially the neuroplasm. Ventral horn neurons have a stronger activity than the dorsal horn neurons. The nucleolus shows negative activity while the nucleus has negligible activity (Figure 21).

The activity of IDPase in protein deficient animals is variable and ranges from a moderate activity in some neurons to a strong activity in others. Glial cells show a strong activity and those closely apposed to neurons with weak activity show a much stronger activity themselves. The neuropil has negligible to mild activity. This localization represents the distribution of the endoplasmic reticulum (Figure 22).

Thiamine pyrophosphate activity appears strong in the motoneurons while a variable activity is noticed in dorsal horn cells. The activity extends considerably into cell processes. The TPPase activity is distributed in relationship to the presence of the Golgi complex within the neuroplasm. Dense, darkly stained areas of Golgi network are plentiful in the motoneurons. The glial cells also react strongly to TPPase activity while the neuropil shows a mild reaction.

In malnourished animals, blood capillaries show strong activity. Dorsal neurons have a moderate reaction while motoneurons have from moderate to strong activity. Some sections appear to have lost a considerable amount of deposition but the glial cells have strong activity both in white and gray mater.

Esterase

Acetylcholinesterase activity in healthy monkeys ranges from mild in certain neurons through moderate in some to strong in a substantial number of others. This strong activity is located primarily in the lateral column, especially in the neuroplasm and cell membrane and processes. Variable activity is noticed in individual motoneurons and is probably related to the functional state of each neuron. Both mild and moderately stained cells are visible in all areas of the spinal cord.

In malnourished animals some reduction in neuroplasmic deposition is indicated, although the activity near cell membranes remains fairly unchanged. The surrounding neuropil has mild activity. Substantia gelatinosa of the exterior horn exhibits a diffuse activity of the enzyme. The neuroglia and ependymal cells appear to have negative activity. No activity is evident in the nucleus or nucleolus.

Dehydrogenases

Succinic dehydrogenase shows moderate to strong activity in the perikaryon of motoneurons in healthy animals while the sensory neurons have primarily moderate activity.

The malnourished group seems to have reduced SDH activity. Even the apparent reduction does not diminish to any great extent the intensity of the activity. More activity exists in the gray than the white mater although glial cells show a moderate reaction. Lateral neurons show mild to moderate staining while the substantia gelatinosa and neuropil show mild activity with a few cells showing a strong

reaction. The glial cells that are closely apposed to chromatolysed neurons have a strong reaction. This may represent some type of metabolic equilibrium between the neuron and surrounding glial cells as a structural functional unit.

Lactic dehydrogenase in the healthy animals is strong within the neuroplasm. There is some difference in the strength of activity between ventral and dorsal horn cells. The nucleus and nucleolus have negative activity. The glial cells exhibit mild activity while neuropil has a moderate reaction. Blood vessels have mild activity in the vascular endothelium. Ependymal cells have mild activity within their boarders but negligible within the cells (Figure 25, 26).

Alpha-glycerophosphate dehydrogenase activity is moderate to strong in the neurons. The nucleus and nucleolus show negative activity. The neuropil also shows moderate activity while glial cells show negligible activity. Both blood vessels and the ependymal cells have mild activity. The ventral neurons show a stronger cytoplasmic activity than do the dorsal horn cells in the healthy animals.

CHAPTER V

DISCUSSION

The physiological alterations produced by a certain degree of protein deficiency are difficult to explain in any satisfactory manner. It probably is easier to explain the physiological deficiency of one defined compound, such as a trace element, in terms of metabolic reactions specifically affected by the deficiency than a more generalized deficiency of protein. It is rather not easy to identify the compound in terms of any one metabolic reaction.

The present study, however, shows a pattern of changes in the morphology of cells that underwent protein deficiencies. This pattern illustrates severe chromatolysis in the neuronal cytoplasm and the enzymes show alterations that may have significant bearings on function. The enzymes changes may affect their ability to mediate important metabolic reactions taking part in adaptation processes to changed protein content in the diet.

Since nucleic acids are of fundamental importance as genetic and intracellular messengers for controlling metabolic processes, the change in their content should reflect a shift in the protein synthesizing mechanism of the nerve cells. Also the duplication of DNA as well as the coding for RNA probably becomes affected to some extent. Ordinarily the body is capable of adapting itself to protein intakes at levels much lower than usually recommended provided that

calorie requirements are supplied by carbohydrates and other nutrients are sufficiently available. The body can still compensate for protein deficiency of fairly drastic proportions by a certain degree of adaptability of the cellular metabolic machinery provided that the deficiency is not of long duration. Severe and long lasting protein deprivation may cause irreversible damage to the biochemical activity within the cellular milieu. During periods of rapid brain growth, protein deficiency, if initiated at such periods, may have devastating effects on the cell size and population finally attained (Dobbing, 1968; Winick, 1969, 1970).

The studies on the spinal cords of young squirrel monkeys show that the neurons respond to deficiency in terms of the individual metabolic activities of each cell. This possibly accounts for variance in degree of chromatin loss in some cells within the malnourished animals. The ribonucleoprotein complex in different cells may be involved in metabolic activities of varying rates and thus explain the different degrees of vacuolation in even adjacent neurons (Olkowski and Manocha, 1972).

As has been noted by a number of workers the differential staining patterns of nerve cells' Nissl substance, represent differences in metabolism and function (Hochberg, 1965; Packenberg, 1967; LaVelle and LaVelle, 1971). The chromalum-gallocyanin stainability may represent the different stages of metabolic function or the result of certain physiological manipulations such a nutrition. While the adult body may utilize small, labile protein reserves, mainly from the

liver, to alleviate severe protein deficiencies, it is questionable if the growing fetus has such reserves from which to draw needed proteins. The maternal diet is thus the main source of protein reserves and in depleted diets, their limited supplies might affect the acquisition of protein precursors needed to synthesize new structural and functional proteins within the fetus or young growing animal.

Although Hydén (1967) indicates that the amount of ribonucleoprotein complex in the neuroplasm changes with the functional state of the neurons, it is doubtful that chromatolysis of this substance in the course of protein deficiency during development has the same implication. As shown by both the hematoxylin-eosin, chromalum-gallocyanin and the Feulgen reaction for DNA; there is a loss of nucleic acids in severe, long lasting protein deficiency. These staining techniques show a lack of positive deposition within certain cells while in others only a mild or moderate amount is indicated. In control animal preparations, these histochemical techniques reveal a substantial abundance of nucleic acids and Nissl's substance deposition. Studies of LaVelle and LaVelle (1971) show that Nissl substance contains protein, therefore any lack of protein in diets of young animals may affect the formation of Nissl substance.

The changes that may result from severely chromatolysed neurons can be and probably have far reaching effects. Some cells are pycnotic and the condensation of chromatin may cause drastic functional disorganization of the ribonucleoprotein complex and a complete dis-

ruption of cellular enzyme makeup and activity. Although cells can and do adjust to metabolic changes, it is difficult to predict whether they will or will not adapt indefinitely, particularly to severe protein deficiency.

The observation that in severely chromatolysed neurons, many neuroglia can be seen in close contact or within their vicinity has some implications. It appears that the neurons and glial cells have a dynamic functional relationship, since the latter cells stain not only strongly for nuclear material when chromalysis is indicated within adjacent neurons, but also become plentiful in such situations (Hydén, 1967). The neuroglia therefore may be considered as auxillary metabolic units that are called upon to augment the needs of neurons in pathologic situations.

The PAS-positive reaction for glycogen, especially strong in protein depleted cells, indicates that the energy needs of these cells may be increased. Although the spinal cord and central nervous tissue in general use glucose as the primary source of energy, it must be remembered that glycogen is a polymer of glucose. In protein depleted diets, the amount of carbohydrates is substantial and these animals probably convert the excess glucose into glycogen for storage.

The spinal cord controls simple reflexes which develop during intrauterine growth (Bodian, 1966) and since reflex activity involves energy, the high concentration of glycogen may be explained. Furthermore, the development of these reflexes is dependent on synaptic formation and synthesis of high quality specific enzymatic proteins

together with phosphate esters of adenine. These activities, when taken together, help to generate the energy needed for the function of the neurons. The ability for the system's development depends on the available supply of basically high quality proteins, carbohydrates, lipids and minerals. The impact of the proteins have been assessed in terms of deficiency on growth and development.

The morphological changes observed in this study are similar with findings in malnourished pigs and dogs (Platt and Stewart, 1969, 1971; Stewart and Platt, 1968). Their studies show loss of Nissl substance in motoneurons and increased number of perineuronal oligodendrocytes. This, in addition to the pathology of squirrel monkey neurons as observed in the present study, indicates drastic functional alterations in the cells and their ability to synthesize proteins in a normal manner.

The interpretation of enzyme data, particularly that of oxidative and hydrolytic enzymes is cautious. Although observable differences have been observed in enzymes activity between healthy and malnourished animals, the effects of fixatives on the activity cannot be over emphasized (Manocha, 1970).

The activities of the phosphatases show that certain metabolic activities are initiated rather early in squirrel monkey development. Thiamine phyophosphatase activity established the involvement of the Golgi complex. It is apparent that the enzyme has a variable reaction under different experimental conditions, including malnutrition (Olkowski, et. al., 1972). The Golgi complex has varied roles in the neuron, such as synthesis or packaging of polysaccharides or both (Peterson and

Leblond, 1964), condensation of proteins synthesized on ribosomes (Caro and Pallade, 1964; Nadler, et. al., 1964), or the formation of membranes (Whaley, et. al., 1964). The apparent strong activity in protein depleted animals is difficult to understand. Normally neurons have a high rate of protein synthesis in the well developed endoplasmic reticulum. The decrease in Inosine diphosphatase which is located at the subcellular level of the Golgi complex-endoplastic reticulum-lysosomes system (Novikoff, 1967) must be significant although the implications are not known. Acid phosphatase is located within the lysosomes in malnourished and healthy animals. Koenig (1969) indicates that acid hydrolases within the lysosomes are in a latent state and can be called upon to split hydrolytically most cellular macromolecules. It is possible that in long protein depletion, the activity of these hydrolases is triggered and their release splits mucopolysaccharides. This excessive activity may affect proper protein transport after synthesis has occurred in the ribosomes (Manocha and Olkowski, 1972). The ATPase activity seems to be reduced in depleted animals possibly because of the slow down of metabolic activity within the neurons of these animals.

Succinic dehydrogenase of the Kreb's cycle has been investigated since the cycle is responsible for a significant source of free energy in higher animals as primates. These enzymes are located primarily within the mitochondria and show a reduction in activity in the severely malnourished state. It appears a compensatory role is indicated by glia adjoining chromatolysed neurons since these show rather strong

activity. It seems as though the role of glia in severe malnutrition is limited, inspite of their apparent role in the energy requirements. Hydén (1967) has stated that "the neuron and its glia are interlocked in an energy system,which may swing between the two positions."

The apparent increased lactate dehydrogenase is probably an indication of increased activity in anaerobic metabolism but as in the case of the variable distribution of α-glycerophosphate dehydrogenase, this interpretation is only speculative.

Acetylcholinesterase distribution implicates acetylcholine metabolism in the spinal cord. The enzyme activity is not significantly different in either malnourished or healthy animals at the membrane areas. This may mean impulse transmission is not severely affected by malnutrition.

Conclusions

The implication of this study of protein depletion during intrauterine growth of fetal squirrel monkeys is significant in that certain histopathological alterations have been noticed. These alterations bring to bear certain biochemical adaptions but in severe derangement of ribonucleoprotein complex or in that or chromatin, it is likely that the fetuses can no longer withstand such drastic changes.

The fetuses are not protected from any adverse conditions of nutrition that affect the mother during pregnancy. From the histochemical and cytochemical studies, it is obvious that the degree to which fetuses can be subjected to both morphologic and functional disorders is severe. If these effects are not corrected in infancy,

they can have serious consequences in later life, particularly for the human being.

CHAPTER VI

RECOMMENDATIONS

The study of prenatal protein deficiency in squirrel monkeys shows the spinal cord as a highly vulnerable organ to protein restriction. The experimental protein diets fed were started about 45 days after pregnancy and it would be interesting to find out whether malnutrition at the onset of pregnancy has the same or a more severe consequence on the spinal cord or other nervous tissue. The use of histo- and cytochemical techniques for *in situ* localization helps to pinpoint any variation in the distribution of nucleic acids, ribonucleo-protein complex, proteins, enzyme distribution and other cellular constituents within the spinal cord neurons.

Such a problem appears interesting and I recommend that more work be done in this area.

Table 1

Enzyme Cytochemical Methods Used

Enzyme	Techniques	References
Acid phosphatase (AC)	Simultaneous coupling azo-dye	Barka and Anderson (1963) Gregg and Pearse (1952) Barka (1960)
Adenosine triphosphatase (ATPase)	Lead method	Wachstein and Meisel (1957)
Alkaline phosphatase (AK)	α-naphthyl phosphatase method, Naphthol AS-Bi phosphate azo-dye	Gomori (1951, 1952) Burstone (1958 a,b)
Inosine diphosphatase (ATPase)		Novikoff and Goldfischer (1961)
Thiamine pyrophosphatase (TPPase)		Novikoff and Goldfischer (1961)
Acetylcholinesterase (AChE)	Direct coloring thiocholine, Koelle and Friedewald (1949)	Karnovsky and Roots (1964) Coupland and Holmes (1957)
Alpha-glycerophosphate dehydrogenase (α-Gly-P DH)	Nitro BT method	Hess et al. (1958)
Lactic dehydrogenase (LDH)	Nitro BT method	Hess et al. (1958)
Succinic dehydrogenase (SDH)	Nitro BT method	Nachlas et al. (1957)

Table 2

Cytochemical Observations on the Enzyme Activities in Healthy and Malnourished Animals

Enzymes	25% Protein Histochemical Reaction				8% Protein Histochemical Reaction				4% Protein Histochemical Reaction			
	Ventral horn cells	Dorsal horn cells	Glial cells	Neuropil	Ventral horn cells	Dorsal horn cells	Glial cells	Neuropil	Ventral horn cells	Dorsal horn cells	Glial cells	Neuropil
Acid phosphatase	+++	++ to +++	+	+	++	++	±	± to +	++	+	±	± to +
Alkaline phosphatase	±	±	±	++	±	±	±	++	±	±	±	+
Inosine diphosphatase	+++	++ to +++	+++	++	++	++	+++	++	+	±	+	±
Thiamine pyrophosphatase	+++	++ to +++	+++	+	++ to +++	++	+++	+	± to +	± to +	± to +	±
Adenosine triphosphatase	++ to +++	++ to +++	+++	++	++	++	+++	++	++	++	+++	++
Acetylcholinesterase	+++	+++	±	+	++ to +++	++	±	+	++ to +++	++	±	+
Succinic dehydrogenase	+++	++ to +++	++	++	++ to +++	++ to +++	+ to ++	+	++	++	+	+
α-glycerodehydrogenase	++ to +++	++	±	++	++	++	±	+	+	+	±	±
Lactic dehydrogenase	+++ to ++++	+++	+ to ++	++	+++	+++	+ to ++	+	++ to +++	++ to +++	+ to ++	+

Legend: - = negative reaction
± = negligible activity
+ = mild activity
++ = moderate activity
+++ = strong activity
++++ = very strong activity
N = nucleus
Nu = nucleolus
C = cytoplasmic activity

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