CHAPTER II

MATERIALS AND METHODS

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The present studies were concerned mainly with the effects of prenatal or neonatal nutritional deficiencies on brain glutamate dehydrogenase (GDH) and decarboxylase (GAD). Additional determinations were made of protein and DNA as well in some experiments. Records were kept of body weight changes during treatment and terminal brain weight. Food intake was recorded wherever possible.

For the studies on prenatal nutritional deficiency females weighing 200 to 250 g were fed either a low protein diet (5%) or a high protein diet for a period of one month and allowed to mate with males from the stock colony. The pregnant rats were separated as soon as pregnancy was evident and kept in individual cages. They were continued on the deficient diet till partus. Neonatal undernutrition was induced by either feeding the mothers a low protein diet during lactation or by increasing litter size. For the studies on the effects of protein deficiency confined to the lactation period the mothers were fed a low protein diet from partus. For the studies on neonatal undernutrition induced by increasing litter size, pups born on the same day were pooled and kept with foster mothers in litters of 16 or 8. For the studies on the effects of maternal protein deficiency during both gestation and lactation the mothers were started on the

low protein diet before mating and continued on this diet till the end of the experiment. In all these cases a control group of mothers was fed a high protein diet throughout. Water was provided ad lib. and so was food except when food restriction was part of the experimental procedure.

Except in experiment IIa on the effects of neonatal undernutrition, the Charles Foster strain obtained from the Sarabhai Research Centre, Baroda was used (Strain B). In this experiment which was done earlier, a slow growing strain obtained previously from Haffkine Institute and used till recently in this laboratory was used (Strain A).

The animals were fed either a stock diet or the experimental diets specified. The composition of these diets is shown in Tables 1 and 2.

Table 1 : Composition of the stock diet.

Ingredient	Amount (g)
wheat flour	350
bajra flour	100
bengalgram flour	110
milk powder	210
sprouted pulses	160
groundnut oil	70
dark green leafy vegetables	60-80

Table 2 : Composition of low and high protein diets.

	% dietary protein		
	4	5	20
washed casein	4.8	6.0	24.0
vitamin mixture	2.0	2.0	2.0
salt mixture	4.0	4.0	4.0
groundnut oil	7.0	7.0	7.0
sucrose	-	18.0	-
sago	82.2	63.0	63.0

Shark liver oil 2-3 drops per week per rat providing 70-100 mcg of vitamin A was given.

Edible casein obtained from Amul Dairy, Anand was washed with alcohol and then washed free of alcohol with tap water and finally with distilled water. The washed casein was dried and used. Each lot was analysed for nitrogen content by the microkjeldahl method and protein content was calculated as N x 6.25.

Commercially available sago prepared from tapioca flour (Manihot utilissima) was ground and used in place of starch as it proved more suitable. It contains only 0.2 per cent protein and no more than traces of vitamins and minerals.

As tapioca flour is processed to some extent during the preparation of sago, the starch in the same is believed to be readily available (Booher, Behan and McMeans, 1951).

The vitamin mixture used was formulated previously in this laboratory on the basis of the allowances suggested by Brown and Sturtevant (1949), the recommendations made by NAS-NRC (1962) and evidence reviewed by Mitchell (1964). The composition of the same is given in Table.3

Table 3 : Composition of the vitamin mixture.

	Amount per kg of the diet
thiamine hydrochloride (mg)	1.5
riboflavin (mg)	2.5
pyridoxine hydrochloride (mg)	1.0
niacin (mg)	15.0
calcium-d-pantothenate (mg)	10.0
choline chloride (mg)	750.0
inositol (mg)	200.0
para-aminobanzoic acid (mg)	10.0
folic acid (mg)	1.0
cyanocobalamin (mcg)	5.0
biotin (mcg)	1.0

powdered sugar approximately 19 g so as to make a total of 20 g.

The salt mixture used was the Hawk-Oser salt mixture No. 3 (Hawk, Oser and Summerson, 1954). The composition of the same is given in Table 4. Both mixtures were prepared in bulk and stored in air tight bottles. The vitamin mixture was stored in brown bottles in cold condition.

Table 4 : Composition of the salt mixture.

	Amount (g)
salt mixture A*	16.7
dicalcium tricitrate, $4H_2O$	308.2
$Ca(H_2PO_4)_2\ H_2O$	112.8
$K_2H\ PO_4$	218.7
KCl	124.7
NaCl	77.0
$CaCO_3$	68.5
$MgSO_4$ (anhydrous)	38.3
$3MgCO_3.\ Mg(OH)_2\ 3H_2O$	35.1

* 100 g of salt mixture A contained :
$FeNH_4$ citrate, USP, 91.41 g; $CuSO_4.5H_2O$, 5.98 g; NaF, 0.76 g; $MnSO_4.2H_2O$, 1.07 g; $KAl\ (SO_4)_2.12H_2O$; 0.54 g; and KI, 0.24 g.

The diets were prepared once a week and the vitamin mixture and groundnut oil were added at the time of feeding.

Chemicals

The chemicals used in the experiments were of research grade purity and obtained either from the British Drug House or the Sarabhai Chemicals. The fine chemicals were obtained from the sources indicated below :

L-glutamic acid	BDH
Pyridoxal phosphate	E.Merck
Gamma-aminobutyric acid	BDH
NAD reduced	V.P.Chest Institute, Delhi
2-oxoglutarate	Sigma
Ninhydrin	Pfizer
Bovine Albumin	Armours

The experiments carried out are briefly described below :

Experiment I

This experiment was concerned with the effects of prenatal undernutrition. As mentioned earlier, females were fed a low protein diet from a month before mating through gestation till partus. The progeny of these animals, were compared with those born of mothers normally nourished throughout.

Experiment IIa and IIb

In previous studies in this laboratory undernutrition during the first four weeks of life was found to decrease the activities of brain glutamate dehydrogenase and decarboxylase

(Parameswaran, 1974). These studies raised a question as to the age when the effects of neonatal undernutrition are most evident as this might be expected to depend on the rate of brain growth and maturation of brain enzymes. The present studies were carried out to study the effects of neonatal undernutrition at different ages.

Neonatal undernutrition was induced by manipulating litter size. Animals were reared by foster mothers either in large or standard litters of size 16 and 8. The pups were killed at 7, 14, 21 and 28 days after birth. Some pups were killed soon after birth for comparison.

The above experiment as well as previous experiments in this laboratory were carried out on a strain of rats (strain A) used in this laboratory for the last 15 years. This strain was obtained from the Haffkine Institute, Bombay and reported to be originally of the Wistar strain.

The possibility that the strain had undergone some changes during the several decades prior to their introduction in this laboratory cannot be ruled out. Animals in this strain reached a weaning weight of 45-50 g at 4 weeks of age. More recently a faster-growing strain (Charles foster strain B) which reaches a weight of 45-50 g at 3 weeks was obtained. As the older strain has more or less been replaced by this

strain for studies currently in progres, the above experiment was repeated with the new strain.

Experiment III

In the previous experiment undernutrition induced during the neonatal period by increasing litter size was found to be associated with deficits in GDH and GAD at 14 and 21 days, but not at seven days. In other studies in this laboratory it was found that the effects of prenatal and neonatal undernutrition on brain lipids varied with the severity of undernutrition (Rajalakshmi and Nakhasi, 1974). These observations raised the question as to whether the effects of neonatal undernutrition on brain GDH and GAD show a similar variation. In this connection increasing litter size to 16 is found to result in a body weight deficit of about 50% whereas a much greater deficit of about 70% is obtained by feeding the mothers a low protein diet during both gestation and lactation. Studies were made of the effects of increasing the severity of undernutrition during the neonatal period by this procedure on the size of the brain enzyme deficits observed previously.

Experiment IV

In the experiments just described, deficits in brain GDH and GAD were found with 2 and 3 weeks of neonatal undernutrition but not at one week. Studies were therefore made of the

comparative effects of undernutrition confined to different periods prior to weaning i.e. undernutrition confined to only one week (first or third week after birth) or two weeks (first and second or second and third). To restrict undernutrition to the periods specified, pups born of and reared by well-nourished mothers were transferred to mothers fed a low protein diet from partus at the appropriate age. All the pups were sacrificed at weaning.

Experiment V

As mentioned earlier, in previous studies in this laboratory protein deficiency during the immediate postweaning period was found to result in deficits in brain glutamate dehydrogenase and decarboxylase, but undernutrition confined to the postweaning period was without similar effect, but resulted in deficits when it was preceded by neonatal undernutrition (Rajalakshmi, Parameswaran, Telang and Ramakrishnan, 1974). The above observations were sought to be confirmed and extended by comparing the effects of undernutrition and/or protein deficiency before and after weaning and the reversibility of any effects found. In this connection some irreversible effects on behavioral parameters have been found when postweaning undernutrition or protein deficiency on rats is preceded by neonatal undernutrition as well (Barnes et al, 1968).

Pups were reared in small or large litters till weaning. Those reared in large litters were fed for 6 weeks from weaning either a low protein diet (4%) or a high protein diet (20%) ad lib., or the latter in restricted amounts. Pups from the control group were fed a high protein diet throughout. The protein content of the low protein diet and the amount of food provided in the restricted group were adjusted so that body weights remained practically constant during this period. Some animals from each group were killed at the end of the 6 weeks period. The remaining animals were fed a high protein diet ad lib. for a further period of 6 weeks.

Experiment VI

As the effects of deficiency can be expected to vary in different regions of the brain, studies were made of the comparative effects of neonatal undernutrition in the cerebrum, cerebellum and brain stem at 14 and 21 days. Neonatal undernutrition was achieved by feeding a low protein diet to the mothers from the day of partus till the specified period.

Biochemical assays

The animals were decapitated and the whole brain with the olfactory lobes removed, freed from blood vessels, wiped free of blood and weighed. For studies on different regions the brain was placed on a petridish inverted over crushed ice.

The cerebrum was taken along with the frontal lobes and the cerebellum and brain stem were taken up to the first dorsal nerve. Fine forceps and a scalpel were used for the separation. A ten per cent extract was prepared by homogenizing the brain with an appropriate quantity of potassium phosphate buffer (0.02M), pH 7.0 in a Potter Elvehjem homogenizer for 60 seconds at 0° at 4000 r.p.m. Triton-X-100 was added at the level of 2.5 mg per ml of homogenate and the same kept at $0\text{-}1^{\circ}$ for 30 minutes. This homogenate was used as such for the assay of GAD. For the assay of GDH, the supernatant obtained after centrifugation at 8000 x g for 30 minutes at 0° was used. The details of the assay system and procedures used in the estimation of enzyme activities are summarized in Table 5.

Estimation of protein

Protein was precipitated from 0.2 ml of the above extract by adding one ml of 10% trichloroacetic acid. The supernatant was removed by centrifugation and the residue dissolved in 10 ml of 0.1N. Sodium hydroxide and protein content was estimated by the method of Lowry, Rosebrough, Farr and Randell (1951). Bovine albumin was used as standard.

Estimation of DNA

One ml of the above extract was mixed in a tube containing 2.5 ml of 10% TCA and allowed to stand for 15 minutes after

Table 5 : Assay system and procedure for GDH and GAD.

	L-glutamate : NAD oxidoreductase (GDH) (E.C., 1.4.1.2)	L-glutamate-1-carboxy-lyase (GAD) (E.C., 4.1.1.15)
Basis of method used	Bulen (1956)	Rajalakshmi et al (1965)
Buffer	Tris, pH 8.0, 100 micromoles	Potassium phosphate buffer, pH 6.5, 50 micromoles
Substrate	2:oxoglutarate (neutralized), 20 micromoles.	L-glutamate (Neutralized), 10 micromoles.
Enzyme extract	Supernatant, 0.1 ml.	Crude extract, 0.2 ml.
Other components	$(NH_4)_2SO_4$, 300 micromoles, $NADH_2$, 0.1 micromole.	Pyridoxal phosphate, 0.02 micromoles.
Final volume	3 ml.	1.0 ml
Conditions of incubation	30^0, 2 minutes.	37^0, 30 minutes.
Start of reaction	Addition of 2-oxoglutarate	Addition of enzyme extract.
Termination of reaction	-	The assay tubes heated for 2 minutes in a boiling water bath.
Treatment of blank	2-oxoglutarate omitted	Fresh enzyme replaced by boiled enzyme.
Parameter measured	Oxidation of $NADH_2$ measured in terms of reduction in optical density at 340 mu.	Chromatographic determination of GABA formed.
Enzyme unit	Amout of enzyme which catalyses the oxidation of 1 micromole of $NADH_2$ in one minute.	Amount of enzyme required to form 1 micromole of GABA in 1 hour.

which it was centrifuged at 2000 r.p.m. for 10 minutes. The residue was washed with 2.5 ml of 10% TCA, suspended in 5 ml of 95% ethanol and the supernatant was removed by centrifugation. The lipid-free residue was suspended in 2.5 ml of 5% TCA and heated for 15 minutes at 90^{0} with occasional stirring, cooled and centrifuged at 2000 r.p.m. for 10 minutes. The supernatant obtained was taken for the estimation of DNA by the method of Schneider (1957).

In the presentation of the results enzyme activity has been expressed in terms of units per g tissue. The picture remained practically the same when expressed in terms of specific activity as the concentration of protein was not appreciably affected by any of the conditions studied. As DNA concentration was either unaltered or slightly increased this was also true of activity per mg DNA. Since the differences reported would only be magnified by a consideration of whole brain values, tissue concentration is considered a valid criterion.