CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

As stated earlier the present studies were concerned with the effects of protein deficiency or undernutrition on brain glutamate dehydrogenase and decarboxylase during the neonatal and postweaning periods. GABA transaminase was also assayed in the first series of experiments.

In addition estimations were made of liver protein and glutathione in some cases.

Animals

Unless otherwise specified weanling albino rats weighing 40-50g from the stock colony maintained in this laboratory were used. The animals assigned to the different groups were matched for body weight and sex and were usually littermates. They were housed individually in galvanized iron cages. Water and food were provided *ad lib.* unless otherwise specified. Food intake was recorded daily and body weights once a week.

Diets

The composition of the diet was as given in Table 7 for studies involving the manipulation of dietary protein content.

Edible casein, obtained from Amul Dairy, Anand, was washed first 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 calculated therefrom.

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).

Table 7 : Composition of the diet

ingredient	amount (g)
vitamin free casein* + sago flour	87
vitamin mixture	2
salt mixture	4
groundnut oil	7
shark liver oil	2-3 drops per week per rat providing 70-100 mcg. of vitamin A.

* amount adjusted so as to provide protein at the required level.

The vitamin mixture used was formulated previously in this laboratory on the basis of the allowances suggested by Brown and Sturtevant (1949), recommendations made by NAS-NRC (1962) and evidence reviewed by Mitchell (1964). The composition of the same is given in Table 8.

Table 8 : Composition of vitamin mixture.

vitamin	amount per kg. of the diet
thiamine hydrochloride (mg)	1.5
riboflavine (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 amino benzoic acid (mg)	10.0
folic acid (mg)	1.0
cyanocobalamin (mcg)	5.0
biotin (mcg)	1.0
powdered sugar to make a total weight 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 9. Both mixtures were prepared in bulk and stored in air tight bottles. The vitamin mixture was stored in brown bottles at 1-3° in the cold room.

Table 9 : Composition of salt mixture.

ingredient	amount (g)
salt mixture A*	16.7
dicalcium tricitrate, $4H_2O$	308.2
$Ca(H_2PO_4)_2\ H_2O$	112.8
K_2HPO_4	218.7
KCl	124.7
NaCl	77.0
$CaCO_3$	68.5
$3MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$	35.1
$MgSO_4$ (anhydrous)	38.3

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

For studies on neonatal and postweaning undernutrition either the stock diet or a standard diet, composed as described in the appropriate context was used (Experiments VIa and VIb).

The diets to be fed 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 were obtained from British Drug House Limited or from E. Merck. The fine chemicals were obtained from the sources indicated :

L-glutamic acid	E. Merck
Gamma amino butyric acid	BDH
Pyridoxal phosphate	L. Light and Company, London.
2-oxo-glutaric acid, NAD (reduced)	Sigma Chemical Company, U.S.A.
Reduced glutathione	BDH
Ninhydrin	BDH
Bovine albumin	Armour Labs.

Different experiments concerned with different aspects were carried out. They are briefly described below.

Experiments Ia and Ib

Previous studies showed deficits in brain enzymes in animals fed a 5% protein diet as compared to those fed a 20% protein diet. The object of these experiments was to ascertain the minimum level of protein required in the diet to prevent these deficits. Animals were fed diets providing 5,8,10,15 or 20% protein for a period of 10 or 5 weeks.

Experiment II

In the previous experiment brain enzyme deficits were found with 5% protein in the diet but not with 8%. This experiment was carried out to identify more precisely the minimum level of protein needed for the maintenance of normal activities of the brain enzymes studied. Animals were fed diets providing 5,6,7,8 or 20% protein for a period of 5 weeks.

Experiment III

In previous studies in this laboratory the effects of protein deficiency were reversed by a diet providing 20% protein. In experiments I and II 8% protein in the diet was found to be adequate to prevent the effects of deficiency. This experiment was carried out to ascertain the level of

protein needed for reversing the effects of previous deficiency. During the depletion period of 10 weeks groups of animals were fed either a 5% or 20% protein diet. Thereafter the animals fed the 5% protein were divided into five groups and fed 5,8,10,15 or 20% protein for a further period of 10 weeks. The animals in the high protein group were continued on this diet.

Experiment IV

In the previous studies protein deficiency was induced by feeding a 5% protein diet. This experiment was carried out to investigate the effects of more severe degrees of protein deficiency on brain enzymes. Animals were fed 0,1,2,3,4,5 or 20% protein diet for a period of 5 weeks.

Experiment V

In previous experiments the period of treatment was five weeks or more. In this experiment the progressive effects of protein deficiency or deprivation after different periods of treatment were investigated. Animals were fed 0,5, or 20% protein diets for 1,2,3,4 or 5 weeks.

Experiments VIa and VIb

In previous studies in this laboratory protein deficiency but not moderate undernutrition during the postweaning period was associated with brain enzyme deficits which were also

found with undernutrition during the neonatal period. These experiments were carried out to investigate the effects of moderate and severe undernutrition and subsequent rehabilitation on brain glutamate dehydrogenase. The diet used in these experiments consisted of wheat flour, 60g., whole milk powder (buffalo), 40g., groundnut oil, 5 g and common salt, 1 g. Shark liver oil was given orally once a week to provide 70-100 mcg. of vitamin A. The controls were fed ad lib. while the experimentals were fed 50% of the food intake of the control animals in experiment VIa and 33% in experiment VIb. The period of depletion was five weeks in both the experiments. Period of repletion was 11 weeks in experiment VIa and 5 weeks in experiment VIb.

Experiment VII a and VII b

In previous studies in this laboratory neonatal undernutrition induced by increasing the litter size to 16 was found to result in deficits in brain glutamate dehydrogenase and decarboxylase in cerebrum, cerebellum and brain stem (Rajalakshmi and Ramakrishnan, 1969a). The object of the present experiments was to investigate the effects of neonatal undernutrition in the whole brain and their reversibility with postweaning rehabilitation. Additional studies were carried out to determine whether the effects of postweaning deficiencies are influenced by the prior nutritional status of the animals.

Groups of pups were reared in standard or large litters of 8 and 16. In experiment VIIa animals reared in standard or large litters were fed either ad lib. or in restricted amounts for a period of 5 weeks from weaning. In experiment VIIb animals reared in standard or large litters were fed a 5 or 20% protein diet or the latter in restricted amounts for a period of 10 weeks.

Biochemical assays

At the end of treatment the animals were killed by decapitation. The whole brain with the olfactory lobes was removed, freed from blood vessels, wiped free of blood and weighed. A ten per cent extract was prepared in potassium phosphate buffer (0.02M), pH 7.0, by homogenizing the brain in a Potter-Elvehjem homogenizer for 60 seconds at 0^{o} at 4000 rpm. The homogenate was treated with triton X-100 at the level of 2.5 mg/ml and kept at $0-1^{o}$ for 30 minutes. This homogenate was used as such for the assay of GAD and GABA-T. For the assay of GDH the supernatant obtained after centrifugation at 8000 x g for 30 minutes at 0^{o} was used. The details of the assay system and procedures used in the estimation of enzyme activities are summarized in Table 10.

Estimation of glutathione

Immediately after slaughter, the liver was removed, wiped free of blood and weighed. The nitroprusside method of Grunert

Table 10 : Assay system and procedure for GDH, GAD and GABA-T in brain.

	L-glutamate : NAD oxidoreductase (GDH) (E.C., 1.4.1.2)	L-glutamate-1-carboxy-lyase (GAD) (E.C., 4.1.1.15)	4-aminobutyrate : 2-oxoglutarate amino-transferase (GABA-T) (E.C., 2.6.1.19)
Basis of method used	Bulen (1956)	Rajalakshmi et al (1965)	Rajalakshmi et al (1965)
Buffer	Tris, pH 8.0, 100 micromoles	Potassium phosphate buffer, pH 6.5, 50 micromoles.	Tris, buffer, pH 8.0, 50 micromoles
Substrate	2:oxoglutarate (neutralized), 20 micromoles.	L-glutamate (neutralized), 10 micromoles.	2:oxoglutarate (neutralized), 10 micromoles.
Enzyme extract	Supernatant, 0.1 ml.	Crude extract, 0.2 ml.	Crude extract, 0.2 ml.
Other components	$(NH_4)_2 SO_4$, 300 micromoles, $NADH_2$, 0.1 micromole.	Pyridoxal phosphate, 0.02 micromoles.	4-aminobutyrate, 10 micromoles; pyridoxal phosphate, 0.02 micromoles.
Final volume	3 ml.	1.0 ml.	1.0 ml.
Conditions of incubation	30^o, 2 minutes.	37^o, 30 minutes.	37^o, 30 minutes.
Start of reaction	Addition of 2-oxoglutarate	Addition of enzyme extract.	Addition of enzyme extract.
Termination of reaction	-	The assay tubes heated for 2 mts. in a boiling water bath.	The assay tubes heated for 2 mts. in a boiling water bath.
Treatment of blank	2-oxoglutarate omitted.	Fresh enzyme replaced by boiled enzyme.	Fresh enzyme replaced by boiled enzyme.
Parameter measured	Oxidation of $NADH_2$ measured in terms of reduction in optical density at 340 mμ.	Chromatographic determination of GABA formed.	Chromatographic determination of glutamate formed.
Enzyme unit	Amount 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.	Amount of enzyme required to form 1 micromole of glutamate in 1 hour.

and Phillips (1951) was used with slight modifications. A portion of the liver was used to prepare a ten per cent extract in cold 3% metaphosphoric acid using a Potter-Elvehjem homogenizer. The solution was then saturated with sodium chloride and filtered. The filtrate obtained was used for the estimation of glutathione. To 2.0 ml of the filtrate were added 6 ml of saturated sodium chloride solution. After equilibration at 20° for 5-10 minutes, 1 ml of sodium nitroprusside solution and 1 ml of alkaline sodium cyanide reagent were added in quick succession and the resulting colour intensity measured at 540 mμ within one minute in a Klett-Summerson colorimeter. Two ml of 2% metaphosphoric acid saturated with sodium chloride was used for blank. Reduced glutathione was used as standard.

Estimation of protein

A 10% extract of liver tissue was prepared in 0.9% KCl and 0.2 ml of this was precipitated with a 10% solution of trichloroacetic acid. The residue was washed with one ml of cold 5% trichloroacetic acid, centrifuged and the supernatant removed. The residue was dissolved in 10 ml of 0.1N sodium hydroxide and protein content estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). Bovine albumin was used as standard.