P A R T I I

STUDIES ON THE RELATION BETWEEN DIET AND BRAIN ENZYMES

C H A P T E R I I

MATERIALS AND METHODS

MATERIALS AND METHODS

As stated earlier the present investigations were concerned with the effects of different diets on brain enzymes in young albino rats. Four different experiments concerned with the following aspects were studied.

I. comparative effects of calorie and protein deficiencies during the postweaning period.

II. effects of supplementation of lysine or lysine-rich foods to kodri (Paspalum scorbiculatum L.), maize (Zea mays) and wheat (Triticum aestivum).

III. effects of supplementation of glutamic acid to diets differing in protein content and to a low protein diet at different levels.

IV. effects of vitamin A depletion and repletion in rats given low and high protein diets.

ANIMALS

Weanling albino rats from the stock colony maintained in this laboratory were used in these investigations. The

animals assigned to the different groups were matched for age, body weight and sex. To the extent possible littermates were assigned to the different groups. They were housed individually in galvanised iron cages. Water and food were provided ad libitum unless otherwise specified. Food intake was recorded daily and body weight once a week. The animals were sacrificed for biochemical studies at the end of the treatment.

DIETS

Unless otherwise stated the composition of the diet was as given in Table 1.

Table 1: 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 providing 68-102 mcg of vitamin A

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

Edible casein obtained from Amul Dairy, Anand was washed first with alcohol and then washed free of the latter 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 than commercially available starch. 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.

The vitamin mixture 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 evidences reviewed by Mitchell (1964). The composition of the same is given in Table 2.

Table 2: 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-panthothenate (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)	1.0
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 3. Both mixtures were prepared in bulk and stored in air tight bottles. The vitamin mixture was stored in brown bottles in the cold room.

The diets to be fed were prepared once a week, but the vitamin mixture and groundnut oil were added at the time of feeding.

Table 3: Composition of salt mixture

	amount (g)
salt mixture A*	16.7
calcium citrate, $4H_2O$	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
$3MgCO_3.Mg(OH)_2\ 3H_2O$	35.1
$MgSO_4$ (anhydrous)	38.3

*100g of salt mixture A contained: $FeNH_4$ citrate, 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.

EXPERIMENT I

As stated earlier, the object of this experiment was to ascertain whether the effects of a low protein diet were due to reduced food intake. Two groups were fed ad libitum

low protein (LP) and high protein (HP) diets containing 5 per cent and 20 per cent protein. A third group was fed the HP diet in amounts consumed by the LP group. The period of treatment was 10 weeks.

EXPERIMENT II

(a) Studies were made of the effects of supplementing kodri (Paspalum scorbiculatum L.). The composition of the diet is given in Table 4. An additional group was fed a diet containing 7.3 per cent casein. The food was given ad libitum and the period of treatment was 10 weeks.

(b) In this experiment, the nutritive value and protein quality of maize (Zea mays), and wheat (Triticum aestivum) were improved by the addition of bengal gram (Cicer arietinum) and fenugreek leaves (Trigonella foenum graecum) and the effects of such addition studied. The composition of the diet is given in Table 5. The animals were compared with a group receiving a 10 per cent casein diet. The animals were fed ad libitum for 8 weeks.

Table 4: Composition of the diets used in Experiment IIa

ingredients (g)	groups 1	2	3
kodri	87.0	87.0	-
lysine	-	0.173	-
casein	-	-	8.0
sago	-	-	79.0
salt mixture	4.0	4.0	4.0
vitamin mixture*	2.0	2.0	2.0
groundnut oil	7.0	7.0	7.0

the protein content of the diet is 7.3 g per 100 g.
*two drops of shark liver oil were given orally to all animals once a week providing 68-102 mcg of vitamin A.

Table 5: Composition of different diets used in Experiment IIb

ingredients	amount (g) basal diet	supplemented diet
cereal or millet*	150	120
bengal gram (Cicer arietinum)	-	30
groundnut oil	8.0	8.0
fenugreek leaves (Trigonella foenum graecum)	-	40
crude common salt	4.0	4.0

*maize (Zea mays) and wheat (Triticum aestivum) were used.

EXPERIMENT III

As mentioned earlier, supplementation with glutamic acid was found in previous studies to reverse the effects of a low protein diet on brain glutamate dehydrogenase and decarboxylase. Further studies were carried out to ascertain the level of supplementation at which such reversal occured and the effects of such supplementation to diets differing in protein content.

(a) Glutamic acid was added at the level of 5 per cent to diets containing 3, 5, 8 and 20 per cent protein. 5 g of glutamic acid were added to 100 g of diet before feeding.

(b) Glutamic acid was added to a low protein diet (5%) at a level of 1,2,3,4 or 5 per cent and studies were made of the effects of such addition. In both the above experiments the rats were fed ad libitum for a period of 10 weeks.

EXPERIMENT IV

Studies were made of the effects of vitamin A deficiency in animals fed low and high protein diets. Additional studies were made of the effects of repletion.

Groups of rats were fed either the LP or HP diet with or without vitamin A. After 11 weeks of treatment all the animals in the LP groups and half the animals in the HP group were killed for the biochemical studies. The remaining animals were given the HP diet with vitamin A for a period of 6 weeks.

The low protein animals failed to show either clinical deficiency symptoms or enzyme deficits after 11 weeks of treatment. The experiment was repeated in ~~in~~ the case of low protein animals with a longer period of treatment (17 weeks).

FOOD MATERIALS

The millets, cereals, pulses, sago and groundnut oil were purchased in bulk from the local market.

A summary of the experimental conditions used in different experiments is given in Table 6. The following parameters were measured in all the above experiments:

1. growth
2. food intake

Table 6: Variable used in different experiments

experiment	experimental manipulation	diets used	mode of feeding	no. of groups	no. of animals in each group	period of treatment (weeks)
I	calorie - restriction as compared to protein deficiency	LP and HP	LP and HP groups fed ad libitum; a third group on HP diet pair fed with LP group	3	7	10
II	glutamic acid supplementation	3,5,8 and 20% protein with and without glutamic acid	ad libitum	14	4-8	10
III	quality of protein	(a) kodri with and without lysine supplement; 7.3% casein diet	ad libitum	3	7	10
		(b) maize and wheat with and without bengal gram and green leaves; 10% casein diet	ad libitum	3	14	8
IV	vitamin A omission	LP and HP with and without vitamin A	ad libitum	8	8	11 and 17

3. liver weight
4. brain weight
5. brain enzymes
   (a) glutamate dehydrogenase
   (b) glutamate decarboxylase.

In addition determinations of brain GABA-T and liver GDH activities were made in experiment I.

BIOCHEMICAL ASSAYS

At the end of treatment the animals were killed by decapitation. The brain was removed and freed from the blood vessels. It was weighed and a 10 per cent extract was prepared in potassium phosphate buffer (0.02 M), pH 7.0, by homogenizing in a Potter-Elvehjem homogenizer for 60 seconds at 0° at 4000 rpm. The homogenate was treated with triton X-100 at a level of 2.5 mg/ml and kept at 0-1° 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° was used. The details of the assay system and procedures used in the estimation of enzyme activities are summarized in Table 7.

Table 7: Details of enzyme assay

	glutamate dehydrogenase (GDH)	glutamate decarboxylase (GAD)	GABA transaminase (GABA-T)
basis of method	Bulen (1956)	Rajalakshmi, Govindarajan and Ramakrishnan (1965)	
buffer	tris, pH 8.0, 100 micromoles	potassium phosphate buffer, pH 6.5, 50 micromoles	tris, pH 8.0, 50 micromoles
substrate	2-oxoglutarate (neutralised), 20 micromoles	L-glutamate, 10 micromoles	2-oxoglutarate, 10 micromoles
enzyme extract	0.1 ml supernatant	crude extract, 0.2 ml	crude extract, 0.2 ml
other components	$(NH)_2SO_4$, 300 micromoles $NADH_2$, 0.1 micromole	pyridoxal phosphate, 0.02 micromole	GABA, 10 micromoles, pyridoxal phosphate, 0.02 micromole
final volume	3.0 ml	1.0 ml	1.0 ml
conditions of incubation	30°, 60 seconds	37°, 30 minutes	37°, 30 minutes
start of reaction	addition of 2-oxoglutarate	addition of enzyme extract	addition of enzyme extract
termination of reaction	-	the assay tubes heated in boiling water bath for 2 minutes	the assay tubes heated in boiling water bath for 2 minutes
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 by reduction in optical density at 340 mμ at 30 seconds interval	GABA formed	glutamate formed
enzyme unit	amount of enzyme which catalyses the oxidation of 1.0 micromole of $NADH_2$ in 1 minute	amount of enzyme required to form one micromole of GABA in one hour	amount of enzyme required to form one micromole of glutamate in one hour

CHEMICALS

The chemicals used in the experiments were of research grade purity and were generally obtained from the British Drug House Ltd. or from E. Merck except for the following chemicals, which were obtained from the sources indicated.

Chemicals	Sources from which obtained
GABA 2-oxoglutaric acid NAD reduced Pyridoxal phosphate	Sigma Chemical Co., U.S.A.
DEAE cellulose	Carl Scheichel and Schuell, New Hampshire
Fluphenazine dihydrochloride Trifluprομazine Hydroxyzine	Unichem Laboratories Ltd. Bombay
Chlorpromazine	May and Baker Ltd., Bombay

Calcium phosphate gel (dry wt. 11 mg per ml) was prepared by the method described by Keilin and Hartree (1938).