C H A P T E R II

MATERIALS AND METHODS

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As stated earlier the present investigations were aimed at a study of the distribution of selected metabolites and enzymes in different regions of the rat brain and changes in the same with protein deficiency during the post-weaning period.

ANIMALS

Albino rats from the stock colony were used in the investigations. They were 4-5 weeks old at the start of treatment. They were fed either a low protein diet (LP) containing 5.5% casein or a high protein diet (HP) containing 22% casein. The animals assigned for the two groups were matched for age, body weight, sex and littermates. They were housed two to a cage and provided food and water ad libitum. Food intake and body weights were recorded once a week. The animals were fed on the diets specified for a period of 10 weeks at the end of which they were killed for biochemical studies.

LOW PROTEIN (LP) AND HIGH PROTEIN (HP) DIETS

The LP and HP diets were composed as shown in Table 5.

Table 5: Composition of LP and HP diets

	amount (g) per 100 g	
	LP	HP
vitamin free casein	5.5	22.0
vitamin mixture	2.0	2.0
salt mixture	4.0	4.0
groundnut oil	7.0	7.0
sago flour	81.5	65.0
shark liver oil	2 drops per rat per week (provided 200-300 i.u. of vitamin A)	

Edible casein obtained from the Amul Dairy, Anand was washed free of vitamins with alcohol and then washed free of the latter with distilled water. The washed casein was dried and used. Each lot was analysed for nitrogen content by microkjeldhal method and protein content calculated therefrom. The same was found to be about 90% so that the LP and HP diets provided 5-6% and 19-20% protein respectively.

Sago prepared from tapioca was used in place of starch as it proved more suitable than commercially available starch. It contains only 0.3% protein and no more than traces of vitamins and minerals. As the

tapioca flour is processed to some extent during the preparation of sago, the starch in the same is believed to be available. The performance of these animals compared with that of animals fed cooked diets composed of wheat and other ingredients.

The vitamin mixture was formulated in this laboratory on the basis of 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 6. The salt mixture used was the Hawk-Oser salt mixture no. 3 (Hawk, Oser and Summerson, 1954). Both mixtures were prepared in bulk and stored in airtight bottles in the cold room.

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

CHEMICALS

The chemicals used in the experiments were of research grade purity and were obtained from The British Drug Houses Ltd. or from E. Merck.

The fine chemicals were obtained from the sources indicated:-

Table 6: Composition of vitamin mixture

vitamin	amount per kg of the diet
thiamine hydrochloride (mg)	1.5
riboflavin (mg)	2.5
pyridoxine hydrochloride (mg)	1.0
niacin (mg)	15.0
Ca-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

chemicals	sources from which obtained
L-glutamic acid, cysteine hydrochloride, ascorbic acid	E. Merck
DL-alanine, hydroxylamine hydrochloride, ninhydrin	BDH Ltd.
DL-aspartic acid	Eastman Kodak Co.
bovine albumin	Armour Labs.
L-glutamine, GABA, glutathione (reduced), 2-oxoglutaric acid, ATP (Na salt), NAD (reduced), sodium pyruvate, pyridoxal phosphate	Sigma Chemical Co.

PARAMETERS MEASURED

The parameters listed below were measured in different regions of the brain.

I. weight and moisture content

II. protein content

III. activities of selected enzymes concerned with glutamate metabolism namely,

1. glutamate dehydrogenase
2. glutamate decarboxylase

3. alanine aminotransferase
4. aspartate aminotransferase
5. D-glutamyl transferase
6. glutamine synthetase

Their systematic names and Enzyme Commission (E.C.) numbers are shown in Table 7. As GABA-transaminase was not affected by protein deficiency (Rajalakshmi et al., 1965) no studies were made of this enzyme.

IV. oxygen consumption of slices and homogenate of different regions of rat brain with glucose and glutamate as substrates

V. ascorbic acid and glutathione.

They had to be measured on different batches of animals as shown below because of restricted availability of tissue and technical considerations

batch	parameters measured
1	weight and moisture content
2	protein and GDH
3	GAD, GPT and GOT
4	glutamyl transferase and glutamine synthetase
5	ascorbic acid
6	glutathione

Table 7: Enzymes assayed in the investigations

trivial name	abbreviation used	systematic name and E.C. number
glutamate dehydrogenase	GDH	L-glutamate: NAD oxidoreductase (deaminating), 1.4.1.2
glutamate decarboxylase	GAD	L-glutamate 1-carboxy-lyase 4.1.1.15
alanine aminotransferase	GPT	L-alanine: 2-oxoglutarate aminotransferase, 2.6.1.2
aspartate aminotransferase	GOT	L-aspartate: 2-oxoglutarate aminotransferase, 2.6.1.1
D-glutamyl transferase	-	glutamine: D-glutamyl glutamyltransferase, 2.3.2.1
glutamine synthetase	-	L-glutamate: ammonia ligase (ADP), 6.3.1.2

For similar reasons several batches had to be used for the studies on oxygen consumption in different regions as shown below:

batch	regions studied
1	cerebellum, medulla and pons
2	midbrain, olfactory lobes and visual cortex
3	hippocampus, basal ganglia and residual brain
4	hypothalamus and corpus callosum.

In addition, data were obtained on body weight and hemoglobin content of blood in the first batch.

HEMOGLOBIN

Blood was collected using Cushnie's tail vain technique (Porter, 1957) and analysed for hemoglobin by the oxyhemoglobin method of Evelyn and Malloy (1938).

DISSECTION OF THE BRAIN FOR STUDIES ON REGIONAL DISTRIBUTION

Soon after decapitation, the skull was opened and the brain removed taking care to avoid damage. The adhering blood vesicles were removed and kept in a petri dish kept in ice.

The different parts of the brain were separated with fine bent forceps and a scalpel. The optic nerves along with the optic chiasma were dissected off from the ventral side of the brain and discarded. The hypothalamus was then removed. The olfactory lobes were also removed from the same side. The part containing the cerebellum, medulla and pons was cut out and the three regions separated. The cerebrum was then turned to the dorsal side and the visual cortex removed.

The remaining dorsal cortex along the cerebral fissure was scraped off and the corpus callosum excised. The cortical layers were then stretched from both sides, and the hippocampus and basal ganglia were excised. The midbrain containing the colliculi was separated. The portion left over after the above separations were also analysed and is referred to as 'the residual brain'. This portion contained the thalamus and parts of the dorsal cortex. The separation of the parts from one brain took about 5 minutes. All these operations were carried out in a cold room maintained at 3 $\pm$ 1°C. Nomenclature and anatomical distinctions of the regions are as described by Zeman and Innes (1963).

In the case of several regions tissue from a number of animals had to be pooled together for one estimation. To facilitate this the required number of brains were removed one by one and kept in ice till they were further dissected. However, for studies on the activities of glutamyl transferase, glutamine synthetase and oxygen consumption separation of the regions was carried out as soon as each brain was removed. The regions separated for respiration studies were kept in the salt solution which will be described latter.

BIOCHEMICAL ASSAYS

Unless otherwise stated the studies were carried out with a phosphate extract. An extract containing 100 mg of the tissue per ml was prepared in a 0.02M potassium phosphate buffer, pH 7.0. The tissue was homogenized in a Potter-Elvehjem homogenizer for 60 seconds at 0° at 4000 rpm.

The activity of GDH was assayed on the supernatant. The phosphate extract prepared above was centrifuged at 6000 X g at 0° in a Servall refrigerated centrifuge for 30 minutes and the supernatant was collected for the enzyme assay.

Glutamyl transferase and glutamine synthetase were estimated using a potassium bicarbonate extract. The extract was prepared using 0.02M potassium bicarbonate as grinding medium at the concentration of one ml per 100 mg of tissue used. The above extract was diluted 10 times with the extracting medium for the assay of glutamyl transferase.

Details of the assay systems and procedure used in the estimation of enzyme activities are summarized in Table 8. In the case of GAD, GPT and GOT the products formed were measured according to the circular chromatographic technique described by Gothoskar, Raina and Ramakrishnan (1961) using butanol:water:acetic acid (40:7:5) as the solvent system.

MEASUREMENT OF OXYGEN CONSUMPTION IN VITRO

For the measurement of oxygen consumption in vitro the standard Warburg technique described by Umbreit, Burris and Stanffer (1964) was followed. The calcium-free mineral salt solution used was the same as that used by Krebs (1950). This salt solution was preferred as the respiration rate is not affected significantly for more than one hour (Krebs, 1950).

Table 8: Assay system and procedure

	glutamate dehydrogenase	giutamate decarboxylase
basis of method used	Bulen (1956)	Rajalakshmi *et al*. (1965)
buffer	tris, pH 8.0, 100 micro-moles	K-phosphate, pH 6.5, 50 micro-moles.
substrate	2:oxoglutarate, 20 micromoles	L-glutamate, 10 micromoles
enzyme extract	0.2 ml (supernatant)	0.2 ml (crude extract)
other components	$(NH_4)_2SO_4$, 300 micromoles, $NADH_2$, 0.1 micromole	pyridoxal phosphate 0.02 micro-moles
final volume	3.0 ml	1.0 ml
conditions of incubation	30°; 2 minutes	37°; 60 minutes
start of reaction	addition of 2:oxoglu-tarate	addition of enzyme extract
termination of reaction	-	the assay tubes were heated in a boiling water bath for 3 minutes
treatment of blank	2:oxoglutarate omitted	fresh enzyme replaced by boiled enzyme
parameter measured	oxidation of $NADH_2$ measured by the reduction in optical density at 340 mu at 30 seconds intervals for 2 minutes	chromatographic determination of GABA 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 glutamate in one hour

Table 8 (contd.)

	alanine aminotransferase	aspartate aminotransferase
basis of method used	Rajalakshmi *et al*. (1965)	Rajalakshmi *et al*. (1965)
buffer	Na-phosphate, pH 7.0, 50 micromoles	Na-phosphate, pH 7.0, 50 micromoles
substrate	pyruvate (Na-salt), 10 micromoles	2:oxoglutarate, 10 micromoles
enzyme extract	0.2 ml (crude extract)	0.2 ml (crude extract)
other components	glutamate, 10 micromoles, pyridoxal phosphate, 0.02 micromoles	aspartate, 10 micromoles, pyridoxal phosphate, 0.02 micromoles
final volume	1.0 ml	1.0 ml
conditions of incubation	37°, 60 minutes	37°, 60 minutes
initiation of reaction	addition of enzyme extract	addition of enzyme extract
termination of reaction	the assay tubes were heated in boiling water bath for 3 minutes	the assay tubes were heated in boiling water bath for 3 minutes
treatment of blank	fresh enzyme replaced by boiled enzyme	fresh enzyme replaced by boiled enzyme
parameter measured	chromotographic determination of alanine formed	chromotographic determination of glutamate formed
enzyme unit	amount of enzyme required to form 1 micromole of alanine in one hour	amount of enzyme required to form 1 micromole of glutamate in one hour

contd...

Table 8 (contd.)

	glutamyl transferase	glutamine synthetase
basis of method used	Tursh (1964)	Gothoskar, Raina and Ramakrishnan (1960)
buffer	acetate, pH 5.5, 100 micromoles	tris, pH 7.2, 80 micromoles
substrate	glutamine, 20 micromoles	glutamate, 100 micromoles
enzyme extract	0.2 ml (crude extract)	0.5 ml (crude extract)
other components	$MnCl_2$, 15 micromoles; ATP (Na salt), 1.25 micromoles; neutralized hydroxalamine hydrochloride, 50 micromoles; $NaHAsO_4$, 20 micromoles	$MgSO_4$, 20 micromoles, neutralized hydroxylamine hydrochloride, 50 micromoles; neutralized cysteine hydrochloride, 20 micromoles; ATP (Na salt), 10 micromoles
final volume	2.2 ml	2.2 ml
conditions of incubation	37°, 60 minutes	50°, 60 minutes
start of reaction	addition of enzyme extract	addition of ATP
termination of reaction	0.8 of ferric chloride reagent	0.8 ml of ferric chloride reagent
treatment of blank	glutamate omitted	glutamate omitted
parameter measured	colorimetric determination of glutamyl hydroxamic acid formed	colorimetric determination of glutamyl hydroxamic acid formed
enzyme unit	amount of enzyme required to form 1 micromole of glutamyl hydroxamic acid in one hour	amount of enzyme required to form 1 micromole of glutamyl hydroxamic acid in one hour

The separated regions were placed immediately in petri dishes containing the cold salt solution and kept on ice. Free hand slices were prepared with the fine edge of a blade. Care was taken to keep the tissue in moist condition.

The sliced material in the salt solution was quickly wiped with filter paper moistened with the salt solution and weighed on a torsion balance. About 100 mg of the wet tissue (50 mg in the case of corpus callosum and hypothalamus) were transferred to the main compartment of the Warburg reaction flask containing the salt solution and 15 micromoles of the substrate where so specified. The total volume of substrate plus the salt solution was 3 ml. 0.2 ml of 6N potassium hydroxide was kept in the centre well of the reaction flask with a small strip of filter paper. The flask was then attached to the manometer and equilibrated at 37° for 10 minutes with atmospheric air as gas phase. At the end of this period the tap was closed and the initial reading was noted. The readings were taken every 15th minute for one hour, the manometers being shaken continuously during this period.

The same procedure was followed for the measurement of oxygen consumption with homogenates except that the tissue

was homogenized with salt solution in a Potter-Elvehjem homogenizer for 60 seconds at 4000 rpm. One ml of the homogenate containing 100 mg of tissue was added in the main compartment of the Warburg reaction flask.

In these experiments nearly 45 minutes elapsed between the cutting of the first animal and the placement of the tissues in the Warburg apparatus as tissues from several animals had to be pooled together.

The oxygen uptake of the tissue slices or homogenates was calculated in terms of microlitres of oxygen utilized per 100 mg of fresh tissue per hour.

PROTEIN

0.2 ml of the 10% phosphate extract of the tissue was added to 0.8 ml cold 10% trichloroacetic acid. The precipitated protein was centrifuged and the supernatant decanted off. The residue was washed with one ml of cold 5% trichloroacetic acid, centrifuged and the supernatant decanted off. The residue was dissolved in 10 ml of 0.1N sodium hydroxide and used for estimating the protein content by the method of Lowry, Rosebrough, Farr and Randall (1951). Bovine albumin was used as standard.

GLUTATHIONE

The nitroprusside method of Grunert and Philips (1951) was used with slight modifications. A 10% tissue homogenate was prepared in a Potter-Elvehjem homogenizer using 3% cold metaphosphoric acid as grinding medium. 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 was added followed immediately by the addition of 1 ml of alkaline sodium cyanide reagent and the resulting colour intensity measured within one minute in the Klett-Summerson colorimeter using 54 no. filter. Two ml of 2% metaphosphoric acid saturated with sodium chloride were used for blank. Reduced glutathione was used as standard.

ASCORBIC ACID

Colorimetric estimation of the total ascorbic acid was done according to the method of Roe (1954).

A CRITIQUE OF THE TECHNIQUES USED

The methods described are subject to some unavoidable limitations. The small size of the rat brain weighing

1.5-2.0 g necessitates the pooling of tissues from several animals for each measurement. Further the weight of different regions varies ranging from 20 mg in the case of hypothalamus to 758 mg in the case of residual brain. Consequently, the number of animals used for each estimation varied from region to region as shown in Table 9. Thus the different estimations were not necessarily done on the same number of animals.

Because of the need for pooling tissues from different animals, some time interval elapsed between the killing of the first animal and the availability of the tissue for biochemical measurements. This varied from one to three hours. Changes in composition and metabolic activity during this period were sought to be minimized by carrying out all the operations in the cold. Further it was presumed that any changes taking place would be similar in both groups so that comparisons of the two groups would be valid. However, this assumption might not be fully justified if factors such as differences in membranal structure influence such changes. It is suspected that this might have happened to some extent in the case of studies on oxygen consumption and the assays of GAD and glutathione. However, these difficulties cannot be avoided in the case of studies on enzymes and oxygen consumption.

Table 9: Number of animals used for each estimation

region	number of animals used for		
	enzymes and protein	respiration and glutathione	ascorbic acid
cerebellum	2	3	1
medulla	4	6	2
pons	4	5	2
midbrain	4	5	2
olfactory lobes	4	6	2
visual cortex	4	5	2
hippocampus	4	5	2
basal ganglia	4	5	2
hypothalamus	8	15	4
corpus callosum	8	15	4
residual brain	1	1	1

Some overlapping of different regions during their separation cannot also be ruled out. Certain of these regions are clearly defined anatomically and could be dissected with high accuracy. These regions include, for example, the cerebellum and the olfactory lobes. In the case of other regions, for example, visual cortex, hypothalamus and the corpus callosum, where an overlapping with other regions is possible, some tissue at the periphery was left out if necessary in order to avoid contamination from the surrounding regions. For the determination of the weight and moisture content, the regions however, were separated as fully as possible. The visual cortex separated was considered to be pure grey matter and the corpus callosum the pure white matter for purposes of separation.

Because of the small size of the regions such as hypothalamus and corpus callosum, the results are in some cases based on a small number of estimations. But the values in each case are based on at least 8 animals so that the small number of estimations is expected to be offset by the use of a large number of animals in each case.

The enzyme assay systems used in the present studies were primarily based on those used in other studies in

this department (Rajalakshmi et al., 1965; 1969; Jacob, Patel and Ramakrishnan, 1967). In the case of GDH, GOT and glutamine synthetase the values obtained were much lower than those reported in the literature, as can be seen from Table 10. In the case of GDH this is believed to be because of the addition of Na CN or ADP in the assay system (Salganicoff and DeRobertis, 1965; Balazs, Dahl and Harwood, 1966).

In the case of glutamine synthetase, this might be because, in the present studies, the blank consisted of the complete system without substrate whereas in other studies only ATP was omitted from the system in the blank. The endogenous production of substrate might account for the low values obtained in the present studies.

In the case of GOT in the present studies the glutamic acid formed was determined chromatographically, whereas in other studies colorimetric determination of the keto acid formed was used. Even so the large difference in values is difficult to explain.

In the case of GAD and GPT, the values were within the range reported by other investigators.

Table 10: Comparison of enzyme techniques and activities of enzyme

GDH (units/g/min)	2.3	5.2	19.7
source	present study	Salganicoff and De Robertis(1965)	Balazs *et al.*(1966)
difference in procedure	not treated with triton X-100 and 2:oxoglutarate blank	assay system contained NaCN and nicotinamide	triton X-100 treated enzyme extract, enzyme blank and ADP added
GAD (units/g/hr)	28	27	26
source	present study	Baxter and Roberts (1961)	Salganicoff and De Robertis (1965)
difference in procedure	GABA formed measured	GABA formed measured	GABA formed measured
GPT (units/g/hr)	34	42	56
source	present study	Salganicoff and De Robertis(1965)	van Kempen *et al.* (1965)
difference in procedure	chromatographic determination	colorimetric determination	triton X-100 treated enzyme extract and colorimetric determination

contd...

Table 10 (contd.)

GOT (units/g/hr)	142	1937	4110
source	present study	van Kempen *et al.* (1965)	Salganicoff and De Robertis (1965)
difference in procedure	chromatographic determination of glutamate formed	triton X-100 treated enzyme extract and colorimetric determination	triton X-100 treated enzyme extract and colorimetric determination
glutamyl transferase	data were not available		
glutamine synthetase (units/g/hr)	12	24	60
source	present study	Salganicoff and De Robertis(1965)	Wu (1963)
difference in procedure	glutamate blank and temperature of incubation 50°C	ATP blank temperature of incubation 37°C	ATP blank temperature of incubation 37°C

In any case the values reported in the literature have shown a very wide variation so that a comparison is difficult. Further the present studies are concerned primarily with a comparison of LP and HP groups and therefore the values are relative than absolute.

The values obtained are consistent with those found in other studies in this laboratory using similar assay conditions. This and the fact that the extent of variation within any one group for any particular region was within reasonable limits give some confidence about the reliability of the measurement made.