CHAPTER II

Methods and Materials

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As mentioned in the introduction, previous studies in this laboratory showed differences between animals fed low and high protein diets with regard to psychological performance and brain enzymes concerned with glutamic acid metabolism. (Rajalakshmi, Govindarajan and Ramakrishnan, 1965). Investigations were therefore conducted (Expt. I) to find out whether the differences observed with a low protein diet on glutamic acid metabolism in the brain can be reversed by the addition of either niacin, pyridoxine or glutamic acid, and increased by the omission of niacin from the vitamin mixture. Nicotinic acid and pyridoxine were chosen as supplements because of their involvement as cofactors in the activities of L-glutamate-NAD-oxidoreductase (E.C., 1.4.1.2) and L-glutamate-1-carboxy-lyase (E.C., 4.1.1.15). Glutamic acid was chosen because of its reported beneficial effects on central nervous function (Waelsch, 1951; Strecker, 1957).

Studies were also made of the effects of the quality of protein on psychological pefformance and cerebral enzymes (Expt. IIa and IIb). Kodri (Paspalum scorbiculatum L.) is a millet lacking in lysine and of poor protein quality consumed by the people in this region. Studies

were made of the effects of supplements of animal and vegetable foods chosen for high lysine content so that they can be expected to improve the protein value of kodri. The supplements used were moth bean (Phaseolus aconitifolius), peas (Pisum sativum), skim milk powder and lysine. Lysine was used in only one of the two experiments carried out.

Pre-school children are the group most subject to protein and other deficiencies in most underdeveloped countries. Worldwide attempts have been made in recent years to combat malnutrition in this age group by formulating and popularising processed food mixtures such as Laubina (Tannous, Cowan, Rinnu, Asfour and Sabry, 1965), Incaparina (Behar and Bressani, 1966), Pronutro (Odendaal, 1966) and processed foods developed at the Central Food Technological Research Institute, Mysore (Parpia, 1966). However, such processed food mixtures are beyond the reach of people in the rural areas in this country because of the increase in cost with processing, and difficulties in transportation and distribution, apart from the gap between production and need. One of the aims of the Applied Nutrition Programme conducted by this department is the formulation and evaluation of meals suitable for children in the post-weaning period based on foodstuffs available to the villager, using processing which can be done by the housewife with simple equipment available at her home. As a first step in

this direction a diet and nutrition survey has been made of children in this age group (Rajalakshmi and Chandrasekharan, 1966). Based on locally available resources and suitability for young children, diets have been formulated so as to correct the basic deficiencies in the home diet. These diets have been fed for breakfast and lunch at the field centre organized by the department (Rajalakshmi and Ramakrishnan, 1966). The customary diets consumed by pre-school children at home and the diet provided at the centre were fed to groups of weanling rats for a period of about fourteen weeks. An additional group was fed the diet which the child would get by taking breakfast and lunch at the centre and dinner at home. The animals were tested on the Hebb-Williams Maze after 6 weeks and sacrificed 14 weeks after testing and their cerebrums assayed for L-glutamate-NAD oxidoreductase, L-glutamate-1-carboxy-lyase, and 4-aminobutyrate-2-oxoglutarate aminotransferase.

Similar studies were also sought to be made of the effects of different supplements to maize (Expt. IV). But unfortunately this experiment happened to be started just before a heat wave, and presumably because of this, the animals in all the experimental groups showed growth arrest, irrespective of the diet they received. The experiment was continued but with the changed objective of investigating

whether such growth arrest in the early period is associated with irreversible brain changes.

Additional studies were made of the effects of electroconvulsive shocks on brain enzymes and behaviour in rats fed low and high protein diets (Expt. V).

The variables used in the different experiments and the parameters measured are shown in Table 4.

In all the experiments young albino rats reared and bred in the laboratory and weighing 40-50 g. at start were used except for experiment IV in which animals weighing between 45-60 g. were used. The experimental groups were matched for age, weight and sex. Littermates were assigned to the different groups to the extent possible.

The animals were caged individually in galvanized iron cages. Water was given ad libitum. Food intake was recorded daily and body weight once a week. Other experimental conditions used are summarized in Table 5.

In experiments II, III and IV the animals were tested for psychological performance after dietary treatment for the period specified and killed for the biochemical studies. In experiment V the animals were subjected to a foot shock followed by electroconvulsive shocks after dietary treatment

Table 4

Varibles used in the different experiments

Experiment	Independent variable	Parameters used		
		Behavioural measure	Body composition	Cerebral enzymes
I	Dietary protein content and the effects of different supplements	-	Weight gain	
II	Dietary protein quality	Performance on the water maze	Weight gain, blood hemoglobin, nitrogen retention, liver protein	L-glutamate-NAD-oxidoreductase (E.C. 1:4:1:2)
III	Diets fed to rats based on the diet provided to pre-school children at a rural play centre	Error scores on the Hebb-Williams maze	Weight gain, blood hemoglobin	L-glutamate-1-carboxy-lyase (E.C. 4:1:1:15)
IV	Growth arrest caused by heat in animals fed maze diet with and without supplements	Visual discrimination and reversal learning	Weight gain, blood hemoglobin, nitrogen retention, liver protein.	4-aminobutyrate-2-oxoglutarate aminotransferase (E.C. 2:1:1:19)
V	Electroconvulsive shocks to animals fed low and high protein diets.	Shock avoidance	-	

Table 5

Summary of the experimental conditions used in different experiment

Expts.	No. of groups	No. of animals in each groups	Diets used	Salt mixtre used	Vitamin mixture used	Mode of feeding	Period of treatment before testing (weeks)	Period of testing (weeks)	Total period of treatment before sacrifice (weeks)
Ia	6	6	Low protein with and without different supplements (Table 6)	Hawk, Oser salt mixture No.3	Formulated in the laboratory (Table 8)	ad libitum	25	-	25
Ib	6	5	- do -	- do -	- do -	Pair feeding	6	-	6
IIa	4	6	Kodri with and without different supplements (Table 7)	Formulated in accordance with the salt content of the diet (Table 9)	-	ad libitum	38	-	38
IIb	5	6	- do -	- do -	Formulated in the laboratory (Table 8)	Pair feeding	5	1	6
III	3	14	Diets fed to rats based on the diet provided to pre-school children (Table 10)	-	-	ad libitum	6	8	14
IV	6	10-15	Maize diet with and without different supplements (Table 11)	Hawk, Oser salt mixture No.3.	Schultz, 1950.	- do -	27	3	30
V	6	6	Low and high protein diet and stock diet(Tables 6 & 12).	- do -	Formulated in the laboratory (Table 8)	- do -	11	1	12

for the period specified. Half the animals were used for psychological studies and the other half killed for biochemical studies fifteen minutes after the electro-convulsive shocks.

The diets used in the different experiments are given in Tables 6, 7, 10, 11 and 12. The compositions of the salt mixture and vitamin mixture formulated in the laboratory are given in Tables 8 and 9.

The vitamin mixture was formulated in accordance with recommendations made by Brown and Sturtevant (1949) and after taking into consideration the studies reviewed by Mitchell (1964).

The salt mixture was formulated in accordance with the composition of the Hawk Oser salt mixture and the salt content of the diet.

Details not covered above are given below:-

Experiments IIa and IIb

The kodri diets in expt. IIa were fed ad libitum without the addition of a vitamin mixture as the studies were designed from the standpoint of practical nutrition. The second experiment in this group (Expt. IIb) was designed to control both these factors. The animals were pair fed

Table 6

Composition of the diet for experiments Ia and Ib

	Dietary group					
	1 5% casein	2 5% casein -niacin	3 5% casein +niacin	4 5% casein + pyri-doxine	5 5% casein + gluta-mic acid	6 20% casein
Sago (g) (metroxylon sago)	84.0	84.0	84.0	84.0	79.2	69.0
Vitamin free casein (g)	5.0	5.0	5.0	5.0	5.0	20.0
Salt mixture (Hawk; Oser, 1955).	4.0	4.0	4.0	4.0	4.0	4.0
Niacin (mg)	-	-	4.0	-	-	-
Glutamic acid (g)	-	-	-	-	4.8	-

Peanut oil and vitamin mixture were added before feeding at 0.8 ml and 1.0 ml respectively per 10 g. of the diet. In addition 3 drops of shark liver oil per rat were given twice a week. The composition of the vitamin mixture is given in Table 9.

For group 2, niacin was omitted from the vitamin mixture. For group 4, 4 mcg of extra pyridoxine hydrochloride was given per rat per day.

Table 7

Composition of the kodri diets used for experiments IIa and IIb

	Group				
	I	II	III	IV	V
Kodri (Paspalum scorbiculatum L.) (g)	100	60	60	60	100
Skim milk powder	-	9.2	-	-	-
Moth bean (Phaseolus aconifolius Jacq.) (g)	-	-	14	-	-
Peas (Pisum sativum) (g)	-	-	-	16	-
Sago (Metroxylon sago) (g)	-	30.8	26	24	-
Lysine hydrochloride (mg)	-	-	-	-	173
Salt mixture (g)*	2	2	2	2	2
Protein content(g)	8.3	8.3	8.3	8.3	8.3

Peanut oil was added before feeding at 0.8 ml per 10 g. of the diet. In addition 3 drops of shark liver oil per rat were given twice a week.

*The composition of the salt mixture is given in Table 8. Group V was introduced in experiment IIb.
The animals in experiment IIb received 0.5 ml of vitamin mixture (Table 9) per 10 gm. of the diet.

Table 8

Composition of the salt mixture used in experiments IIa and IIb

Calcium citrate	308.0 g.
Calcium carbonate	137.0 g.
Calcium phosphate (mono basic)	56.0 g.
Sodium chloride (common salt)	80.0 g.
Ferric ammonium citrate. U.S.P.	9.0 g.
Copper sulphate	0.59g.
Manganese sulphate	0.11g.

Table 9

Composition of the vitamin mixture

Thiamine hydrochloride	(mg)	1.5
Riboflavin	(mg)	2.5
Pyridoxine hydrochloride	(mg)	1.0
Niacin	(mg)	5.0
Calcium-d-pantothenate	(mg)	10.0
Choline chloride	(mg)	500.0
Folic acid	(Mg)	1.0
Inositol	(mg)	200.0
Sugar	(g.)	19.28

This mixture was dissolved and made up the volume to 100 ml with water.

Table 10

Dietary intake of children fed at the centre and controls not attending the centre

I Dietary intake of children fed at the centre

Menu	Cooked weight (g)	Dry weight (g)	Ingredients
Food provided at the centre:			
Milk	100	10	Skim milk powder 10g.
Conjee	200	55	Wheat 10g.+Bengalgram 10g.+Peanut 20g. + Jaggery 15g.
Mid morning drink:			
Drumstick leaf tea	100	8	Wet leaves 10g. + Jaggery 7g.
Lunch:			
Cereal and Legume Preparation	200	100	Wheat 50g. + Bengal gram 50g.
Leafy vegetables	30	3	Amaranth 15g. + Fenugreek 15g.
Potato	30	5	Potato 20g.
Buttermilk(diluted)	75	2	Skim milk powder 2g.
Carotene rich fruits	30	3	Papayya etc. 30g.
Food taken at Home:			
Tea (Two times)	300	28	Milk 75g.+Sugar 20g.
Chapaties	75	50	Bajra 50g.
Legume preparation	25	5	Redgram 5g.
Vegetables	20	2	Brinjal or other vegetables 20g.

Table 10 (contd.)

II. Dietary intake of children not attending the centre

Menu	Cooked weight (g)	Dry weight (g)	Ingredients
Tea (two times)	300	28	Milk 75g.+Sugar 20g.
Chapaties	75	50	Bajra 50g.
Rice cooked with legume	150	50	Rice 20g.+Kodri 20g. +Red gram 5g.+Oil 5g.
Vegetables	60	6	Brinjal 30g.+Onions 30g.
Legume preparation	50	10	Red gram 10g.
Chapaties	75	50	Bajra 50g.
Vegetables	20	2	Brinjal or other vegetables 20g.

III. Composition of the diet

	Whole day home diet I	Diet at centre +diet at home II	Diet at centre III
Cereals	140	110	60
Pulses	65	65	60
Pea nut	1.5	20	20
Leafy vegetable	5	30	30
Other vegetable	80	70	40
Sugar	20	20	0
Jaggery	0	22	22
Skim milk powder	0	12	12
Whole milk	75	75	0
Vegetable oils	10	10	5

Table 10 (contd.)

IV. Composition of 100g. of the diet (on dry weight basis)

	I	II	III
Cereals	70 (a)	40 (c)	33.5
Pulses	7 (b)	23 (d)	33.5
Peanuts	0.7	7	13.5
Leafy vegetables	0.2	1	1.0
Other vegetables	4.8	3	2
Sugger	9	8	0
Jaggery	0	7	10
Vegetable oil	5	4	3.5
Skim milk powder	0	4	6
Whole milk powder	4	3	0

V. Nutrient content of the diets* (100g. of the diet)

	I	II	III
Calories	360	390	400
Protein(g)	10.5	14.0	15.5
Calcium (mg)	114	290	300
Iron (mg)	7.4	8.0	10.0
Vitamin A (I.U.)	220	1150	1590
Riboflavin (mg)	0.22	0.41	0.42

(a) Bajra(Pennisetum typhoideum):Rice(Oryzasativa):Kodri (Paspalum scorbiculatum L.) 5:1:1.
(b) Bengal gram (Cicer arietinum):Red gram(Cajanus cajan)7:1.
(c) Wheat(Triticum aestivum):Bajra:Kodri:Rice.2:1:0.5:0.5.
(d) Red gram

Common salt and Spices added consistent with culinary practice.

*Calculated from food tables (Aykroyd, Gopalan and Balasubramanian, 1963).

Table 11

Composition of the diet for experiment IV

	Diet	Amount in 100g. of food mixture					
		Maize (g)	Supplement (g)	Sugar (g)	Protein (g)	Fat (g)	Carbohydrate (g)
I	Maize (g)	100	-	-	10.2	9.1	60.1
II	Maize + Bengal gram (Cicer arietinum)	90	5.1	4.9	10.9	9.1	60.2
III	Maize + Skim milk powder	90	3.4	6.6	10.4	9.1	61.4
IV	Maize + Fish flour	90	1.2	6.8	10.1	9.1	61.6
V	Maize + amino acids*	100					
	Lysine (mg)		0.5				
	Tryptophan(mg)		0.4				
	Methionine(mg)		0.2				

1 g. of peanut oil, 0.6 ml of vitamin mixture (Schultz,1950) and 0.4 g. of salt mixture (Hawk, Oser, 1954) were added to 10 g. of the diet. Three drops of shark liver oil per rat were given once a week.

*Amino acids supplemented so as to increase the levels of these amino acids in maize to those given in the FAO reference pattern.

Table 12

Composition of stock diet

Wheat flour	50.0 g.
Pulse mixture (sprouted) consisting of:-	
bengal gram (Cicer arietinum)	11.2 g.
cow gram (Vigna catian)	11.2 g.
green gram (Phaseolus mungo)	11.2 g.
moth bean (Phaseolus aconiti folius)	11.2 g.
Skim milk powder	5.0 g.
Peanut oil	6.0 g.
Crude common salt	5.0 g.

with the addition of vitamin mixture.

Experiment III

The different items in the diet were cooked as in the home or in the centre, dried in an electric even at 60° combined in appropriate proportions and fed to the animals. As the object of this experiment was to study the effects of overall improvement in the diet with regard to protein, vitamins and mineral, no salt and vitamin mixtures were given. The diets were fed ad libitum as the food intake in the different groups did not vary very much.

Experiment IV

Dietary treatment:

The composition of the diet for the first five groups was initially maize flour and the same with one or other of the following ingredients; bengal gram flour, skim milk powder, fish flour, and the deficient aminoacids, viz. lysine, methionine and tryptophan. The supplements were made so as to substitute for 10% of maize protein. The mixtures were made isonitrogenous with appropriate additions of sugar where necessary. The composition of the various food mixtures is shown in Table 11.

The food mixtures were fed in the raw form for the first 13 weeks and in the cooked form for the next 7 weeks.

As mentioned earlier the growth performance of the animals at the end of the above period was unsatisfactory. They were therefore switched to the stock-diet so that the effects of inanition on subsequent development and psychological performance could be studied. At this point another group of ten animals from the stock colony weighing the same as the experimental animals were added.

The composition of the stock-diet used for the control group consisted of wheat flour, a mixture of sprouted pulses such as bengal gram, green gram, cow gram and moth beans, skim milk powder and salt. The composition of the stock-diet is shown in Table 12.

After 9 weeks of feeding the stock-diet, 5 animals from each group were taken up at a time for testing on visual discrimination and reversal. They were sacrificed after testing was completed for all the animals.

The animals which had learned the discrimination and reversal were run daily in the apparatus and fed under testing conditions till they were sacrificed.

The course of the experiment is summarized in Table 13.

Food and water were given ad libitum, except during psychological testing, when they were fed once a day for 30 minutes.

Table 13

The Course of the experiment IV

Stage	Dietary treatment	period in weeks	Date
I	Raw maize	13	April 21 - July 21
II	Cooked maize diet	7	July 22 - Sept 7
* III	Stock diet Batch A	9	Sept 8 - Nov 9
	"" Batch B	16	Sept 8 - Jan 2
IV	Psychological testing with stock diet fed once in 24 hrs. for 30 min. Batch A	6	Nov 10 - Dec 3o
	" B	6	Jan. 3 Feb 12

* Five animals from each group were tested in the first batch(A) and the remaining in the second batch(B).

Experiment V

Effects of electroconvulsive shocks on behaviour and glutamic acid metabolism in rats fed low and high protein diets.

Young albino rats weighing 40-50 g. at start and fed low and high protein diets for 11 weeks were used. Additional groups of stock animals matched with age and weight with the experimental groups were used. At the end of treatment the animals were given foot shock followed by electroconvulsive shock either immediately or after 10 seconds or 5 minutes. Half the animals were sacrificed 15 minutes after the electroconvulsive shocks and the remaining half tested for avoidance of shock after 24 hours.

The composition of low and high protein diets were the same as that of 5% casein and 20% casein diets respectively (Table 6). The composition of the stock diet is given in Table 12.

Behavioural testing

Water maze:

The water filled multiple T-maze used for testing the rats was the same as that described by Polidora, Cunningham and Waisman (1966).

Procedure:

The testing procedure lasted for 6 days. On test day 1 each rat received five pretraining trials. A pretraining trial consisted of placing the rat in one end of the 48 in. single straight channel and allowing it to swim to an escape ramp at the other end of the channel.

On test days 2, 3 and 4, rats received five "forward" trials per day through the maze. The same consisted of placing the rat at the starting point in the maze and retrieving it when it reached the terminal point and ascended the ramp positioned at the end of the terminal straight channel of the maze. Each entry of the whole body into blind alleys was counted as an error.

On test days 5 and 6 rats received 5 "reverse" trials per day ~~trials~~ in which the positions of the starting point and the goal in the previous trials were interchanged.

Although the rats were allowed to retrace the maze, a time-limit of 150 sec. was imposed on any one trial. If the rat had not gained exit by 150 sec., it was removed from the water and given the next trial. There was an interval of 10 minutes or more between trials for a given subject.

Hebb-Williams Maze:

Details of apparatus and procedure can be found elsewhere (Rabinovitch and Rosvold, 1951). Briefly the

animal is trained to go from one corner of a square box covered with wire-mesh to the diagonally opposite corner where food can be found. After adaptation to this procedure barriers are introduced in the field according to a set series of patterns. After training to a criterion running time of 60 sec. per ten runs, with the six preliminary patterns, the twelve test patterns are introduced at the rate of one a day and the deviations (errors) made by the animal from the direct route to the goal recorded, the crossing of previously determined lines being counted as an error. The animal is given ten runs on each test pattern so that the total error scores are recorded over 120 runs. The error scores are believed to reflect the intelligence of the animal, the cleverer animal making fewer errors.

Discrimination learning:

The apparatus used for consisted of a simple T-maze with two aluminium gates hinged at the top which could be locked by placing a nail behind them. The patterns to be discriminated were painted on the gates. They were black vertical and horizontal stripes against white back-ground. In this test, the task of the animal is to discover that the door with one of the two patterns consistently leads to food whereas the other is blocked, the position of the correct door being varied according to previously determined random orders taken from Gellerman's (1933) series. The animal is

given ten trials a day on this task and the training continued till eighteen out of twenty correct choices are made during two consecutive days. The number of trials required for reaching this criterion is taken as a measure of discrimination performance.

Reversal learning:

When the animals reach the above criterion, the wrong and right cues are reversed. That is, if horizontal stripes constitute the rewarding pattern first, vertical stripes are now made rewarding. The number of trials required by the animal to learn this "reversal" is recorded. In this connection, the suggestion has been made that reversal learning is a better criterion of learning ability than the conventional discrimination learning procedure (Rajalakshmi and Jeeves, 1965).

Shock avoidance:

The foot shock and the electroconvulsive shock were administered in an apparatus specially designed for this purpose. The apparatus consisted of a chamber $9\frac{1}{4}"$ x $7\frac{5}{8}"$ x $11\frac{1}{2}"$. The front, and the top made of perspex and the back made of aluminium were detachable. The other two sides were made of aluminium. The floor (Grid floor) of the cage was of stainless steel bars connected to a multiple plug connected to a shock generator. At the centre of the grid floor a wooden block (platform) of 3" x 3" x 2" was fixed.

On the first day of testing each animal was kept on the wooden platform from which it was allowed to step down five times and the time taken recorded in an automatic timer. During this period the ears of the rat were clipped with flattened crocodile clips connected to an electrode from an electroconvulsive shock generator. This treatment was continued on second third and fourth days. On the fifth day as soon as the animal stepped down from the platform to the grid floor, a current of 0.2 MA at 350V was applied to the grid floor for 3 seconds causing a foot shock. An electroconvulsive shock (ECS) 35 to 40 MA strong was applied for .02 second to the ears of the rats either immediately or at 10 seconds and 5 minutes after the foot shock.

Fifteen minutes after the electroconvulsive shock half the animals were sacrificed and their cerebrums assayed for the activities of the following enzymes:

I L-glutamate-NAD-oxidoreductase

II L-glutamate-1-carboxy-lyase

III 4-aminobutyrate-2-oxoglutarate aminotransferase

The remaining half were tested for shock avoidance on the following day. The rats were placed on a platform as usual, and if they failed to step down in three minutes, they were removed from the apparatus. The time taken to step down was used as a measure of shock avoidance.

Nitrogen retention

The animals were caged in metabolism cages for nitrogen retention studies. Urine and faeces samples were collected separately for three days and separate determinations made of the nitrogen content of urine, faeces and diet by the micro-kjeldahl method. The difference between dietary intake and urinary and faecal excretion was taken as the amount of nitrogen retained and the same expressed as a percentage.

Hemoglobin estimation

At the end of the experimental period the hemoglobin content of blood, obtained from the tail by Cushnie's tail vein technique (Porter, 1959), was estimated by the acid hematin method (Wintrobe, 1955).

Liver protein

The animals were killed at the end of the experimental period by decapitation. The liver was quickly removed, freed from adhering blood. The tissue was dried at 60° and nitrogen content determined by the micro-kjeldahl method. Protein content is reported as N x 6.25.

Biochemical assays:

The heads were plunged into powdered ice immediately after

decapitation. The cerebrum was quickly removed, blotted on a filter paper and chilled in a watch glass kept in ice (generally 45-50 seconds elapsed between decapitation and removal of the cerebrum). The tissue was weighed and then minced. A weighed portion of the tissue was homogenized with a grinding medium in a Potter-Elvehjem homogenizer for 30 sec. at 0° at 2000 r.p.m. A 10% sucrose (0.25M) homogenate was prepared and used as such for the estimation of the activities of L-glutamate-1-carboxy-lyase (E.C., 4.1.1.15) and 4-aminobutyrate-2-oxoglutarate aminotransferase (E.C., 2.6.1.19).

A 25% phosphate extract (0.02M potassium phosphate buffer, pH 7.0) was prepared and centrifuged at 5900 g. at 0° in a Servall refrigerated centrifuge. The supernatant obtained was used for the estimation of the activity of L-glutamate-NAD-oxidoreductase (E.C., 1.4.1.2).

Enzyme assays:

Preliminary experiments were carried out to derive the optimum conditions for different enzyme assays with respect to the concentrations of enzyme and substrate, period of incubation and pH. The details of the assay systems, the derived optimum conditions and procedures used in the estimation of enzyme activities are summarized in Table 14.

Table 14

Details of assay system and procedure	L-glutamate-NAD-oxidoreductase (E.C., 1.4.1.2)
Basis of method used	Bulen (1956)
Buffer	Tris, pH 8.0, 100 µmole
Substrate	2-oxoglutarate, 20 µmoles
Enzyme extract	0.1 ml (25% tissue extract)
Other components	$NADH_2$ 0.1 µmole; ammonium sulphate 300 µmole. Final volume 3 ml.
Start of reaction	2-oxoglutarate added
Treatment of blank	2-oxoglutarate omitted
Parameter measured	oxidation of $NADH_2$ measured by change in optical density at 340 mµ at 30 second intervals; for 90 seconds.
Enzyme unit	Amount of enzyme which catalyses the oxidation of 1 µmole of $NADH_2$ in 1 hour.

Table 14 continued

Details of assay system	L-glutamate:1-carboxy-lyase (E.C.,4.1.1.15)	4-aminobutyrate:2-oxoglutarate aminotransferase (E.C.,2.6.1.19)
Basis of method used	Rajlakshmi,Govindarajan and Ramakrishnan, 1965	Rajalakshmi,Govindarajan and Ramakrishnan, 1965.
Buffer	Phosphate buffer, pH 6.5 50 µmoles	Phosphate buffer, pH 7.0 50 µmoles
Substrate	L-glutamic acid(neutralized), 10 µmoles	2-oxoglutarate(neutralized), 10 µmoles
Enzyme extract	0.2 ml (20 mg tissue)	0.2 ml (20 mg tissue)
Other components	Pyridoxal phosphate, 0.02 µmole	4-aminobutyrate, 10 µmoles, pyridoxal phosphate, 0.02 µmole
Temparature and period of incubation	37° , 1 hour	37° , 1 hour
Initiation of reaction	Addition of enzyme extract	Addition of enzyme extract
Termination of reaction	Heating in boiling water bath for 2 minutes	Heating in boiling water bath for 2 minutes
Modification for blank	Enzyme boiled before addition	Enzyme boiled before addition
Parameter measured	µmoles of 4-aminobutyrate formed assayed chromatographically using butanol,acetic acid, water (40:5:7) system	µmoles of glutamate formed assayed chromatographically using butanol, acetic acid,water (40:5:7) system
Enzyme units	Amounts of enzyme required to form 1 µmole of 4-aminobutyrate in 1 hour under assay conditions	Amount of enzyme required to form 1 µmole of glutamate in 1 hour under assay conditions.

Chemicals:

The chemicals used in the experiments were of research grade purity and were obtained from British Drug Houses Ltd. or from E. Merck. The fine chemicals used were obtained from the following sources. Dihydro nicotinamide adenine dinucleotide, and pyridoxal phosphate (sodium salt) from Sigma Chemical Co., U.S.A., Gamma-aminobutyric acid from L. Light and Co., London, and 2-oxoglutaric acid from A.G. Fluka, Switzerland.

Food materials:

The cereals and the pulses were purchased in bulk from the local market. Casein and skim milk powder were obtained from Amul Dairy (Anand) and fish powder from the Central Food Technological Research Institute, Mysore.

Glassware:

Glasswares used were of either Pyrex or Corning.

The electroconvulsive shock generator was a gift from Prof. L. Weiskrantz of Cambridge University. The behavioural research equipment used in the shock avoidance performance was obtained from Grason - Stadler Company Inc., U.S.A.