MATERIALS AND METHODS

Experiment-III : Calorie deficiency in the postweaning period.

Neonatal undernutrition was found to retard the biochemical maturation of the brain. This experiment was conducted to investigate whether brain is vulnerable to calorie deficiency in the immediate postweaning period. As mentioned earlier weaned animals were matched for body weight and sex and divided into three groups. Moderate and severe calorie deficiency was induced by feeding a restricted amount of control diet (20% casein diet) to the experimental animals. Control animals were fed ad libitum amount of 20% casein diet, while experimental groups were fed 50% and 33% of the food intake of their pair fed control animals for a period of ten weeks.

Experiment-IV : Severe protein deficiency in the postweaning period.

This experiment was designed to induce severe protein deficiency in the postweaning period. Weaned animals from the stock colony were taken and matched for body weight and sex. They were divided into five groups. Animals were fed 0%, 2%, 10% and 20% casein diet for a period of 5 weeks.

Experiment-V : Chronic protein deficiency in adult age.

This experiment was conducted to investigate the effect of chronically moderate protein deficiency in the adult age. Adult animals of 20 weeks old were taken from stock colony and matched for body weight and sex and divided into two groups. One group was fed low protein (5% casein diet) and the other a high protein (20% casein diet), for a period of 35 weeks.

Experiment-VI : Postweaning vitamin A deficiency.

There are reports that vitamin A deficiency affects the lipid profile (Bhatt and Rama Rao, 1978; Joshi et al, 1982) as well as chemistry of rat brain (Nakhasi et al, 1977). This experiment was carried out to investigate the influence of vitamin on carbohydrate metabolism in the brain. Pups born on the same day were taken from the stock colony along with the dams. The dams were fed stock diet without vitamin A (green leaves) throughout the lactating period. This would enable to have litters with less liver vitamin A storage. Animals weaned at 3 weeks of age were taken from the stock colony and were divided into four groups after matching body weight and sex.

Animals were either fed a low protein (5% casein) or a high protein (20% casein) diet. In each of these groups the experimental animals were not supplied with vitamin A, while control animals were given 2-3 drops of sharkliver oil individually per week as source of vitamin A. This provides 70-100 ug vitamin A per week. The experiment lasted for a period of 9 weeks, when some of the vitamin A deficient animals fed high protein diet started dying.

All animals were of the Charles Foster strain provided from the stock colony of the Biochemistry Department, Faculty of Science, M.S.University of Baroda, Baroda. All animal experiments were conducted in the same department. In all experiments animals weaned at 3 weeks and weighing around 44g were taken from the stock colony. All the animals were housed individually in galvanized iron cages. There was free access to water. Food was provided ad libitum except where food restriction was part of the experimental procedure. Food intake was recorded daily in all experiments except in preweaning undernutrition. Body weight change was recorded once in a week. Animals were fed either a stock diet or the experimental diet specified. The composition of the diets are shown in Tables-5 & 6).

TABLE-6 : Composition of Vitamin Mixture.

Vitamin	Amount per kg of the diet
Thiamin hydrochloride (mg)	4.0
Riboflavin (mg)	4.0
Pyridoxine hydrochloride (mg)	1.0
Niacin (mg)	15.0
Calcium-d-pantothinate (mg)	10.0
Choline-chloride (mg)	750.0
Inositol (mg)	200.0
Para aminobenzoic acid (mg)	10.0
Folic Acid (mg)	1.0
Cyanocoblamin (µg)	5.0
Biotin (µg)	1.0

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

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 microkjeldal method and protein content was calculated as N x 6.25.

Commercially available sago prepared from tapioca flour (Manihot utilissimas) was ground and used as starch source as it contains only 0.2 percent protein and more than traces of vitamin 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 et al, 1951).

The vitamin mixture used as formulated on the basis of the allowances suggested by Brown and Sturtevant (1949), recommendation made by NAS-NRC (1962) and evidence suggested 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 (Hauk, Oser and Summerson, 1954). The composition of the same is given in Table-7. Both mixtures were prepared in bulk and stored in air tight bottles. The vitamin mixture was stored in dark brown bottles in cold conditions.

Chemicals

The chemicals used in the experiments were of research grade purity and obtained from British Drug House Limited, or Sarabhai Chemicals. All the solvents were of reagent grade purchased from the sources indicated. Fine chemicals were obtained from Sigma Chemicals Ltd., U.S.A. or from sources indicated.

Acetic anhydride	-	Merck, India
ADP-Barium salt	-	Sigma Chemicals Ltd., USA
Alodlase	-	"
ATP-Barium salt	-	"
Bovine albumin	-	"
Chloroform	-	Sarabhai Chemicals, India
EDTA-Mg	-	E.Merck, Germany
Fructose-6-phosphate	-	Sigma Chemicals Ltd, USA
Fructose 1,6 diphosphate	-	"
Glucose	-	British Drug House Ltd., India
Glucose-6-phosphate	-	Sigma Chemicals Ltd., USA
Glucose-6-phosphate--dehydrogenase	-	"
Glyceraldehyde-3-phosphate	-	"
Isocitrate (Potassium)	-	"
Lactate dehydrogenase	-	"

NADH	-	VP Chest Institute, India
NADP	-	Sigma Chemicals Ltd., USA
Petroleum ether (40-60)	-	Sarabhai Chemicals, India
Phosphoethanol pyruvate	-	Sigma Chemicals Ltd., USA
Pyruvate (Sodium salt)	-	"
Potassium ferricyanide	-	British Drug House, India
Sodium Citrate	-	"
Sodium succinate	-	Sigma Chemicals Ltd., USA
Trifluoro acetic acid	-	Koch Light, England
Vitamin A acetate	-	E. Merck, Germany.

Biochemical Assays

At the end of each experiment animals were killed by decapitation. The whole brain with olfactory lobes was removed, freed from blood vessels, wiped free of blood and weighed. A 10% extract was prepared in ice-cold potassium phosphate buffer pH 7.0 by homogenising the brain tissue in a Potter Elvejem homogeniser for 60 seconds at 0° at 400 rpm. The homogenate was treated with triton X-100 at the level of 2.5 mg/ml and kept at 0-1° for 30'. The crude homogenate was used for the assay of phosphofructokinase, Aldolase, and succinate dehydrogenase. But for the assay of pyruvate dehydrogenase, the homogenate was treated with Mg EDTA 1.0 mM and used as such. For the assay of other enzymes such as hexokinase, pyruvate kinase, lactate dehydrogenase, Malate dehydrogenase, isocitrate dehydrogenase, Glucose-6-phosphate dehydrogenase and Malic enzymes, the supernatant obtained after centrifugation at 8000 x g in an IEC refrigerated centrifuge for 30' at 0° was used. All the enzymes were assayed on the same day on which animals were sacrificed. Since the number of enzymes were too many to be assayed on the same day they were divided into three groups.

Each group comprised of three to four enzymes. Each group of enzymes was assayed on different days and finally the result was pooled together. They were grouped in such a way that it would be convenient to do the assay together.

Group-I : Hexokinase, Pyruvatekinase and Lactate-dehydrogenase.

Group-II : Phosphofructokinase, Aldolase, Pyruvate dehydrogenase and Succinate dehydrogenase.

Group-III : Glucose-6-phosphate dehydrogenase Isocitrate dehydrogenase (NADP) and Malic enzyme.

The details of the assay system and procedures used in the estimation of enzyme activities are summarised in Tables-8 to 18.

TABLE-8 : Biochemical parameters and methods used in the investigations.

Parameter	Tissue	Basis of the method used
ENZYMES		
1. Hexokinase E.C. 2.7.1.1	Brain	Land et al. (1977)
2. Glucose-6-phosphate dehydrogenase E.C. 1.1.1.49	Brain	Leong et al. (1981)
3. Phosphofructokinase E.C. 2.7.1.11	Brain	Bull et al. (1958)
4. Aldolase E.C. 4.1.2.13	Brain	Sibley and Lehninger (1949)
5. Pyruvate kinase E.C. 2.7.1.40	Brain	Chainy and Kanungo (1978)
6. Lactate dehydrogenase E.C. 1.1.1.27	Brain	Clark and Nicklas (1970)
7. Pyruvate dehydrogenase E.C. 1.2.4.1	Brain	Bennet et al (1966)
8. Isocitrate dehydrogenase (NADP) E.C. 1.1.1.42	Brain	Gromek and Pastuszko (1977)
9. Succinate dehydrogenase. E.C. 1.3.99.1	Brain	DeRobertis (1962)
10. Malate dehydrogenase (NADP). E.C. 1.1.1.40	Brain	Greengard and Jamdar (1971)
11. Vitamin A	Liver	Trifluro acetic acid Method (Neeld and Pearson, 1963).

TABLE-9 : Assay system and procedure for Hexokinase.
ATP : D hexose 6 phosphotransferase
E.C. 2.7.1.1.

Basis of the method	Land et al. (1977)
Buffer - Tris-HCl pH 7.5	150 µmoles
Substrate - Glucose	4 µmoles
Enzyme extract	0.1 ml
Other components :	
$MgCl_2$	40 µmoles
ATP	5 µmoles
NADP	0.2 µmoles
Glucose-6-phosphate dehydrogenase	1 unit
Final volume	3.0 ml
Condition of incubation	30°, 2.5 minutes
Start of reaction	Addition of substrate
Treatment of blank	ATP omitted
Parameter measured	Reduction of NADP in terms of increase in optical density at 340 nm.
Enzyme unit	Amount of enzyme which catalyses the formation of 1 µmolex of NADPH per minute.

TABLE-10 : Assay system and procedure for Glucose-6-phosphate dehydrogenase D-glucose-6-phosphate : NAD oxido reductase. E.C. 1.1.1.49.

Basis of the method	Leong et al. (1981)
Buffer - Tris pH 7.4	200 µmoles
Substrate Glucose-6-phosphate	1 µmole
Enzyme extract	0.2 ml
Other components :	
$MgCl_2$	10 µmoles
NADP	0.2 µmoles
Final volume	3.2 ml
Condition of incubation	30° - 2.5 minutes
Treatment of blank	Substrated omitted
Parameter measured	Reduction of NADP in terms of increase in optical density at 340 nm.
Enzyme unit	Amount of enzyme which catalyses the formation of 1 µmoles of NADPH per minute.

TABLE-11 : Assay system and procedure for Phosphofructokinase ATP : D fructose--6-phosphate 1-phosphotransferase. E.C. 2.7.1.11.

Basis of the method	Bull (1958)
Buffer - Tris pH 9.0	20 µmoles
Substrate fructose-6-phosphate	5 µmoles
Enzyme extract	0.2 ml
Other components :	
$MgCl_2$	5 µmoles
Ammonium phosphate	1 µmole
ATP	5 µmoles
Hydrazine sulphate (neutralised)	50 µmoles
Bovine albumin (2%)	0.1 ml
Aldolase	2 units
Final volume	1.5 ml
Condition of incubation	37° - 60'
Start of reaction	Addition of substrate
Termination of reaction	Addition of 1.0 ml of ice-cold 10% TCA
Treatment of blank	Addition of substrate after terminating the reaction.
Parameter measured	Formation of triosephosphate colorimetrically using 2:4 dinitrophenyl hydrazine.
Enzyme unit	Amount of enzyme which catalyses the formation of 1 µmole of triose-phosphate per minute.

TABLE-12 : Assay system and procedure for Fructose diphosphate aldolase Fructose 1,6 diphosphate D glycerdehyde 3 phosphate lyase.

E.C. 4.1.2.13.

Basis of method	Sibley and Lehninger (1949)
Buffer - Tris pH 8.0	30 µmoles
Substrate - Fructose 1,6 diphosphate	2 µmoles
Enzyme	0.2 ml
Hydrazine sulphate (neutralised)	125 µmoles
Final volume	1.5 ml
Condition of incubation	37° - 30'
Start of reaction	Addition of substrate
Termination of reaction	Addition of 1.0 ml of ice-cold 10% TCA
Treatment of blank	Addition of substrate after terminating the reaction.
Parameters measured	Formation of triosephosphate colorimetrically using 2:4 dinitrophenyl hydrazine.
Enzyme unit	Amount of enzyme which catalyses the formation of 1 µmole of triosephosphate per minute.

Colorimetric estimation of triosephosphate using 2:4 dinitrophenyl hydrazine :

A suitable aliquot (0.2 ml) from the enzyme assay mixture after terminating the reaction was pipetted into a test tube. Water was added to make the volume 1.0 ml. 1.0 ml of 0.75N NaOH was added and the tubes were incubated at 37° for 15'. 1.0 ml of 0.1% dinitrophenyl hydrazine was added to this preincubated mixture and was again incubated for 10'. After the incubation 7.0 ml of 0.75N NaOH was added to each tube and the colour developed was measured at 540 nm on a Klett Summerson colorimeter within 30'. A reagent blank is run without the sample in the same way. A standard graph of known concentration of triose phosphate is worked out. Concentration of triosephosphate (glyceraldehyde 3-phosphate) formed during enzyme assay is calculated from the standard graph of triosephosphate.

Preparation of 2:4 Dinitrophenyl Hydrazine :-

100 mg of dinitrophenylhydrazine was weighed and transferred into a clean conical flask. Dissolve this in 100 ml of 2N HCl. This is stored in amber coloured bottles and is stable indefinitely.

TABLE-13 : Assay system and procedure for

Pyruvate kinase

ATP : Pyruvate phosphotransferase.

E.C. 2.7.1.40.

Basis of the method	Chainy and Kanungo (1978)
Buffer - Potassium phosphate pH 7.0	10 µmoles
Substrate - Phosphoenolpyruvate	0.2 µmoles
Enzyme extract	0.1 ml
Other components :	
KCl	100 µmoles
$MgCl_2$	10 µmoles
ADP	1 µmole
Lactate dehydrogenase (free of pyruvate kinase)	0.6 units
NADH	0.2 µmoles
Final volume	3.0 ml
Condition of incubation	Addition of substrate
Treatment of blank	Substrate omitted.
Parameter measured	Oxidation of NADH measured in terms of decrease in optical density at 340 nm.
Enzyme unit	Amount of enzyme required to oxidise 1 µmole of NADH per minute.

TABLE-14 : Assay system and procedure for
Lactate dehydrogenase
L-lactate NAD:: Oxidoreductase.
E. C. 1.1.1.27.

Basis of the method	Clark and Nicklas (1970)
Buffer - Potassium phosphate pH 7.0	10 µmoles.
Sodium pyruvate	0.1 µmoles
Enzyme extract	0.2 ml
NADH	0.1 µmoles
Final volume	30° - 2.5', 3 ml
Start of reaction	Addition of substrate.
Treatment of blank	Substrate omitted.
Parameter measured	Oxidation of NADH in terms of decrease in optical density at 340 nm.

TABLE-15 : Assay system and procedure for pyruvate dehydrogenase pyruvate lipoate oxido-reductase. E.C. 1.2.4.1.

Basis of the method	Bennett et al (1966)
Buffer - Potassium phosphate pH 8.0	50 µmoles
Substrate - Pyruvate	10 µmoles
Malate	4 µmoles
Enzyme extract	0.2 ml
Other components :	
$MgCl_2$	10 µmoles
KCl	100 µmoles
ATP	5 µmoles
NAD	0.1 µmoles
TPP	0.2 µmoles
Potassium ferricyamide	3 µmoles
Final volume	6.0 ml
Condition of incubation	37° - 20'
Start of reaction	Addition of enzyme extract
Termination of reaction	Addition of 0.5 ml ice-cold 10% TCA
Treatment of blank	Substrate added after terminating the reaction.
Parameter measured	Reduction of ferricyamide measured in terms of decrease in optical density at 420 nm.
Enzyme unit	Amount of enzyme required for the uptake of 1 µmole of oxygen per minute.

Table-15 (Contd.)

Initial reading was taken at 420 nm before the addition of the crude homogenate as the source of enzyme. After terminating the reaction by addition of 10% ice cold TCA, the tubes were centrifuged in a clinical centrifuge at 4000 rpm and the supernatant was taken for the final reading. The difference between the two readings is taken for the calculation of enzyme activity. Results were calculated based on the assumption that a change in optical density of 0.388 at 420 nm is equivalent to 500 mμatoms of oxygen or 250 mμmoles of oxygen.

TABLE-17 : Assay system and procedure for Succinate dehydrogenase. Succinate (oxido) reductase. E.C. 1.3.99.1.

Basis of the method	De Robertis (1962)
Buffer - Potassium phosphate pH 7.5	100 µmoles
Substrate - Sodium succinate	10 µmoles
Enzyme extract	0.2 ml
Other components :	
Potassium ferricyamide	2 µmoles
Potassium cyamide (neutralised)	20 umoles
Bovine albumin 1%	0.1 ml
Final volume	6.0 ml
Condition of incubation	37° for 15'
Start of reaction	Addition of enzyme extract.
Treatment of blank	Substrate added after termination of the reaction.
Termination of reaction	Addition of 0.5 ml ice cold 10% TCA.
Parameters measured	Reduction of potassium ferricyanide in terms of decrease in optical density at 420 nm.
Enzyme unit	Amount of enzyme required for the uptake of 1 µmole of oxygen per minute.

Initial reading was taken at 420 nm before the addition of the crude homogenate as the source of enzyme. The reaction was terminated by the addition of 10% ice cold TCA. The tubes were then centrifuged in a clinical centrifuge at 4000rpm and the supernatant was taken for the final reading. The difference between the two readings is taken for the calculation of enzyme activity. Results were calculated based on the assumption that a change in optical density 0.388 at 420 nm is equivalent to 500 $\mu\mu$ atom of oxygen or 250 $\mu\mu$moles of oxygen. Thus a slight modification was made from the original method in expressing the enzyme units.

TABLE-18 : Assay system and procedure for Malic Enzyme (NADP). E.C. 1.1.1.40.

Basis of the method	Greengard and Jamdar (1971).
Buffer - Tris HCl pH 7.5	50 µmoles
Substrate - Malate	2 µmoles
Enzyme extract	0.2 ml
Other components :	
$MgCl_2$	20 µmoles
Cysteine HCl (neutralised)	10 "
NADP	0.2 µmoles
Final volume	3 ml
Condition of incubation	30° - 2.5'
Start of reaction	Addition of substrate
Treatment of blank	Substrate omitted.
Parameter measured	Reduction of NADP in terms of increase in optical density at 340 nm.
Enzyme unit	Amount of enzyme required to form 1 µmole of NADP per minute.

Estimation of vitamin A in liver :

Vitamin A (Retinol) was determined by the method described by Neeld and Pearson (1963).

Approximately 1.0g of liver was taken in a conical flask containing 15 ml of 5% potassium hydroxide and saponified in a boiling water bath for 45 minutes. After cooling at room temperature, the volume was made upto 15 ml with distilled water. The flask was covered with brown paper in order to protect from light.

5 ml of saponified sample were taken in a stoppered tube to which 2.5 ml of distilled ethyl-alcohol and 5 ml of petroleum ether (40-60) were added. The contents were shaken vigorously and centriguged at 1000 rpm for 2 minutes. 0.5 ml and 1.0 ml aliquots of the ether layer (upper layer) were evaporated under nitrogen or in a vacuum oven at 60°. 0.1 ml each of chloroform and acetic anhydride were added to this. Finally 2 ml of trifluoroacetic acid (TFA) was added and the colour was read exactly at 30 seconds at 620 nm in a Beckman spectrophotometer against a blank containing chloroform. A standard graph was obtained using different concentrations of vitamin A acetate (0.4 - 4.0 IU).

TABLE-19 : Reagents and standards used for Vitamin A estimation.

Name	Method of preparation
Trifluoroacetic acid reagent	1 ml of trifluoroacetic acid was added to 2 ml of chloroform just before use.
Vitamin A standard :	
(a) Stock	5 mg of standard vitamin A in the form of vitamin A acetate (1,000,000 IU/g) were dissolved in 25 ml chloroform and used as stock solution containing 200 IU/ml. 1 ml of this was diluted to get 10 ml with petroleum ether (40-60) to get an intermediate standard containing 20 IU/ml. This is stable for 24 h when refrigerated.
(b) Working	0.2, 0.4 and 0.8 ml of the intermediate standard mixed with 10 ml petroleum ether, so as to get solution containing 4, 8 and 16 IU per 10 ml..