CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

As mentioned earlier, the aim of the present investigations was to study the pattern of chemical maturation of gray and white matter in the rat brain with respect to the concentrations of lipids, 2',3'-cyclic nucleotide-3'-phosphohydrolase (CNP), phosphoethanolamine (PET) and phosphocholine transferase (PCT) and to investigate the effects on the same of undernutrition during the neonatal period and/or nutritional deficiency with regard to either food energy or protein during the postweaning period. Additional studies were also made of the reversibility of these effects, and of the effects of perinatal thiamine deficiency. The studies were also extended to the composition of the whole brain and spinal cord.

Albino rats belonging to the Charles-Foster strain bred and maintained in the departmental rat colony were used for the investigations except for those experiments (Part of V and VI) which were carried out in the laboratory of Prof. Horrocks in Ohio State University for which rats belonging to the Wistar strain and obtained from commercial sources were used.

The parameters studied were :

(a) the composition of whole brain, gray and white matter with regard to moisture, protein, cholesterol, galactolipids, phospholipids, plasmalogens and gangliosides.

(b) the relative contributions of different phospholipid components such as ethanolamine, phosphoglycerides, choline phosphoglycerides, sphingomyelin and inositol and serine phosphoglycerides to total phospholipids.

(c) activities of CNP, phosphoethanolamine and phosphocholine transferases in gray and white matter.

By comparing the composition of whole brain, gray and white matter attempts were made to arrive at estimates of the proportions of gray and white matter, using the values for critical constituents such as galactolipids.

The various experiments conducted on different aspects are briefly indicated in Table 7. Maternal protein deficiency during lactation was induced by feeding mothers a 5% protein diet after partus. Pups born on the same day were pooled together and allotted to the different groups. For the induction of perinatal thiamine deficiency mothers were fed a thiamine free diet from the 14th day of gestation and continued throughout lactation.

The mothers were provided food and water ad lib. The pups had an access to the maternal diet from about the 16th day of age. At the end of the experimental period the animals were killed by decapitation, the brains removed and the white and gray matters separated (wherever appropriate the spinal cord was also removed) and used for analyses.

Table 7 : Experimental details.

Expt. No.	Experiment	Diets used	No. of groups	Age at start and period of treatment	No. of rats used	Parameters measured
1	2	3	4	5	6	7
I. (a)	Age specific changes in the lipid composition of gray and white matter in the rat brain.	Standard laboratory diet (18% protein)	8	2,3,4,6,9,12, 20 and 52 weeks	7-10 per group	Moisture and lipids such as cholesterol, galactolipids, total phospholipids, gangliosides, plasmalogens, EPG, CPG, SM and SPG + IPG.
(b)	Age specific changes in the lipids in the whole brain of rat.		5	2,3,6,9 and 52 weeks	4-6 per group	Moisture and lipids
II. (a)	Effects of neonatal undernutrition on the lipid composition of the rat brain gray and white matter.	Mothers fed a normal diet (18% protein) or a 5% protein diet (LP) during lactation.	2 (controls and undernourished)	birth to 3 weeks	8 per group	lipids

contd...

Table 7 : contd.

1	2	3	4	5	6	7
	(b) Effects of undernutrition, protein deficiency and rehabilitation during the post-weaning period on animals undernourished prior to weaning.	At weaning the pups raised by control mothers were fed for 6 weeks a high protein diet (20% protein diet) (control) and those raised by LP mothers fed either a 4% protein diet (LP) or high protein diet ad lib. (HP) or in restricted amounts (HP-R).	4 (control, LP, HP, HP-R)	3 weeks to 9 weeks	4 in HP-R group and 7 in other groups	Lipids
III.	Effects of perinatal thiamine deficiency on the lipid composition of the brain and spinal cord.	Mothers fed 25% protein diet ad lib. (control) or pair fed with those fed a thiamine deficient diet (experimental) from 14th day of gestation to 21st days of lactation.	3 (control, pair fed, and experimental)	14th day of gestation to 21st day of lactation	4 in pair fed group and 8 in other groups.	Lipids

contd...

Table 7 : contd.

1	2	3	4	5	6	7
IV.	Effects of perinatal thiamine deficiency on the lipid composition of gray and white matter in the brain.	As in expt. III.	3	As in expt. III.		Lipids
V. (a)	Age specific changes in the activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) in gray and white matter.	Rats at different ages raised on a 26% protein diet.	7	14,16,18,21, 30,60 and 90 days.	9-12 per group	Protein and CNP
(b)	Effects of neonatal undernutrition on the activity of 2',3'-cyclic nucleotide phosphohydrolase (CNP) in gray and white matter.	Mothers fed a high protein (26%) diet (control) or a low protein diet (3.5%, undernourished) during lactation.	2 (control, undernourished)	Birth to 3 weeks.	16 per group	Protein and CNP

contd...

Table 7 : contd.

1	2	3	4	5	6	7
	(c) Effects of perinatal thiamine deficiency on the activity of CNP in the CNS.	As in expt. III.	3 (control, pair fed and experimental)	14th day of gestation to 21st day of lactation.	8 in pair fed group and 16 in other groups.	Protein and CNP
VI.	Effects of neonatal under-nutrition on the activities of phospho-ethanolamine (PET) and phosphocholine transferases (PCT) in the isolated micro-somes of rat brain gray and white matter.	As in expt. V(b)	2 (control, undernouri-shed).	Birth to 3 weeks.	16 per group.	Activities, Km and Vmax values for PET and PCT.

The design of the experiments :

The same is already indicated in Table 7. Some additional details are given below :

Experiment - I : Lipid composition of gray and white matter in rat brain at different ages.

Studies were carried out to understand the lipid composition of gray and white matter in rat brain at different ages. For this purpose groups of rats with the age of 2,3,4,6,9,12,20 or 52 weeks were used. In the first 5 groups approximately equal number of male and female rats were used, whereas in the last 3 groups only male rats were used. After noting their body weights the rats were killed by decapitation, the brains were removed , weighed and the gray and white matters (usually a minimum amount of 150-200 mg of each was collected for the study) were separated. The separated gray and white matters were kept in chloroform:Methanol (2:1) mixture at -10^{0} for the extraction of lipids. The extraction and estimations were done within a month after the collection of the samples.

For the comparison, the whole brain was also analysed at selected ages (2,3,6,9 and 52 weeks).

Experiment - II

(a) Effects of neonatal undernutrition on the lipid composition of rat brain gray and white matter.

(b) Effects of undernutrition, protein deficiency and rehabilitation in the postweaning period on the lipid composition of brain gray and white matter of rats undernourished prior to weaning.

To study the effect of preweaning and postweaning undernutrition or rehabilitation, mothers fed the stock diet (18% protein) during gestation were divided into two groups immediately after delivery. One group was continued on the stock diet and the other fed a low protein diet during lactation. The pups from both groups were either killed or weaned at 21 days of age. The pups reared by the stock diet fed mothers were fed high protein (20%) diet after weaning (control) whereas those reared by the low protein diets were fed either a low protein diet (UN-LP) or a high protein diet ad lib. (HP) or the latter in restricted amounts (UN-HP-R). The rats were caged individually and water was given ad lib. Food intake was recorded daily and body weights once a week.

Experiment - III : Effects of perinatal thiamine deficiency on the lipid composition of rat brain and spinal cord.

To study the effects of perinatal thiamine deficiency female rats were fed a high protein diet (25%) either ad lib. (control) or in restricted amounts (pair fed with thiamine deficient group) or a thiamine free high protein diet ad lib. (experimental) from the 14th day of gestation to the 21st day of lactation. The body weights of the pups were recorded once a week. The pups were killed at 21 days of age, the brains and spinal cords removed and used for the determination of lipids.

Experiment - IV : Effects of perinatal thiamine deficiency on the lipid composition of rat brain gray and white matter.

The dietary and other protocol were as in experiment III except the separated gray and white matter from the brain were used for the analysis.

Experiment - V : Effects of age and nutritional deficiencies on the activity of 2',3'-cyclic neucleotide 3'-phosphohydrolase (CNP) in the rat brain gray and white matter.

Studies were carried out to understand the development of CNP in gray and white matter during development of brain. Male rats of the Wistar strain purchased from Holtzman & Co. Indianopolis, USA were used for this study. The rats were killed, the brains removed and gray and white matter separated. Samples from 3-4 brains were pooled together for each estimation.

A 10% tissue homogenate (about 0.5 g tissue was used) was prepared in 0.32 M sucrose in 0.01 M tris-HCl buffer, pH 7.4. 0.5 ml of the homogenate was used for protein and CNP assays.

Undernutrition was induced as described in experiment II except that the controls were fed a 26% protein diet and the undernourished a 3.5% protein diet. Perinatal thiamine deficiency was induced as described in experiment III. The separated gray and white matter from these rats were processed as described above.

Experiment - VI : Effects of neonatal undernutrition on the activities of phosphoethanolamine (PET) and phosphocholine transferases (PCT) in rat brain gray and white matter.

To study the effect of neonatal undernutrition on PET and PCT, the mothers were fed a high protein diet (26%) or a low protein diet (3.5%) during lactation. The pups were killed at 21 days of age, brains removed and gray and white matter separated. Samples from 4 brains were pooled for each estimation. A 10% tissue extract (about 0.5 g tissue was used) was prepared in 0.32 M sucrose in 0.01 M tris-HCl buffer pH 7.4. Microsomes were prepared from the tissue extract and used for the assay of PET and PCT.

Diets

Animals were fed either a normal stock diet prepared in the laboratory or stock diet obtained from commercial sources or the experimental diets prepared in the laboratory. The composition of these diets are shown in Tables 8,9, and 10.

Table 8 : Composition of the stock diet used in the laboratory.

Ingredients	Amount (g)
Wheat flour (Tritium aestivum)	350
Bajra flour (Pennicitum typhoideum)	100
Bengal gram flour (Cicer arietenum)	110
Milk powder*	210
Sprouted cow peas (Vigna catjang)	80
Groundnut oil (Peanut oil)	70
Fenugreek leaves (Trigonella foenum graecum)**	70
Protein content	18%

* Flour sweepings from the Amul Milk Dairy, Anand, containing skim and whole milk powder.

** Fenugreek leaves were used when in season, otherwise vitamin A acetate in oil (5000 I.U.) were added.

Table 9 : Composition of experimental diets.

	% dietary protein			
	4[a]	5[a]	20[a]	25[b]
Washed casein (g)	4.8	6.0	24.0	30.0
Vitamin mixture (g)*	2.0	2.0	2.0	2.0
Salt mixture (g)**	4.0	4.0	4.0	4.0
Groundnut oil (g) (peanut oil)	7.0	7.0	7.0	7.0
Sucrose (g)	20.0	20.0	20.0	20.0
Sago (g)	62.2	61.0	43.0	37.0

* composition given in Table 11.

** composition given in Table 12.

a. used in experiment II.

b. used in experiments III, IV and V.

Casein used in experimental diets was obtained from Amul Dairy ~~Dicary~~, Anand, washed with alcohol and then with tap water to remove the alcohol and finally with distilled water. The washed casein was dried and used.

Commercially available sago prepared from tapioca flour (Manihot utilissima) was ground and used as a starch source as it contains only 0.2% protein and only 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 (Bocher, Behan and McMeans, 1951).

Table 10 : Composition of commercial diet*.

Ingredients	Low protein (diet)	High protein (diet)
	(g/100 g)	
Casein (vitamin free)	3.5	26.0
Corn starch (85%) + sugar (15%)	81.5	58.9
Hydrogenated cotton seed oil	8.0	8.0
Salt mixture, USP XIV	4.0	4.0
Vitamin mixture	3.0	3.0

* used in experiments V and VI in the form of a pellet.

The vitamin mixture whose composition is given in Table 11 is formulated 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 vitamin mixture was stored at 4° in air tight dark brown bottles.

For thiamine deficient groups (experiments III, IV and V) thiamine hydrochloride was omitted from the vitamin mixture.

Table 11 : Composition of the vitamin mixture*.

	Amount per kg. diet
Thiamine hydrochloride (mg)	4.0
Riboflavin (mg)	4.0
Pyridoxine hydrochloride (mg)	1.0
Niacin (mg)	15.0
Calcium-d-pantothenate (mg)	10.0
Choline chloride (mg)	750.0
Inositol (mg)	200.0
Paramino-benzoic acid (mg)	10.0
Folic acid (mg)	1.0
Cyanocobalamin (mcg)	5.0
Biotin (mcg)	1.0
Cholecalciferol (mg)	7.5

Powdered sugar (approximately, 19g) was added so as to make a total of 20 g.

* used in experiments II, III, IV and V.

For thiamine deficient groups (experiments III, IV and V) thiamine hydrochloride was omitted from the vitamin mixture.

The salt mixture used was the Hawk-Oser salt mixture No.3 (Hawk, Oser and Summerson, 1954). The composition of the

of the same is given in Table 12. Both mixtures were prepared in bulk and stored in air tight bottles.

The diets were prepared once a week and the vitamin mixture and groundnut oil were added at the time of feeding.

Table 12 : Composition of salt mixture*.

	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
$MgSO_4$ (anhydrous)	38.3
$3Mg$ $CO_3Mg(OH)_2$ $3H_2O$	35.1

* used in experiments II, III, IV and V.

** 100 g of salt mixture A contained :

$Fe(NH_4)$ citrate, USP - 91.41 g; $CuSO_4$, $5H_2O$, 5.98 g; NaF, 0.76 g; $MnSO_4$, $2H_2O$, 1.07 g; $KAl(SO_4)_2$ $12H_2O$, 0.54g; and KI, 0.24 g.

Chemicals :

The chemicals used in the experiments were of research grade purity and obtained either from the British Drug House Limited or Sarabhai Chemicals. All the solvents were of reagent grade purchased from the local markets. The following chemicals were obtained from the sources indicated against them.

Adenosine 2',3'-cyclic phosphate Alkaline phosphatase Bovine serum albumin DEOT	Sigma Chemical Co., U.S.A.
Cerebrosides	V.P. Chest Institute, Delhi.
Cholesterol	E. Merck, Germany
^{14}C-CDP-choline	Amersham, U.K.
^{14}C-CDP-Ethanolamine	New England Nuclear, U.S.A.
CDP-Choline CDP-Ethanolamine Cocarboxylase	Sigma Chemical Co., U.S.A.
Deoxycholate (Sodium salt)	Mathenson Coleman and Bell, U.S.A.
Dicaprin	Sedary Research Chemicals, Canada.

Dithiothreitol	Sigma Chemical Co., U.S.A.
EGTA	
Imidazole	
Perchloric acid (60%)	E. Merck, Germany
Phospholipid standards (EPG, CPG, SM, SPG and IPG)	V.P. Chest Institute, Delhi
Potassium dihydrogen phosphate	BDH, U.K.
Ribose-5-phosphate	Sigma Chemical Co., U.S.A.
Sedoheptulose-7-phosphate	
Silica Gel 'G'	E. Merck, Germany.

The reagents used for various estimations are given in Table 13.

Separation of the brain and the spinal cord :

The rats were decapitated and the skin was incised along the median line. The brains with olfactory lobes were removed immediately and put in cold normal saline (0.9%). The adhering blood vessels were removed by pressing the tissue between the folds of filter paper and the tissue was weighed. For separating the spinal cord, the dorsal muscles were cut and the vertebral column cut open from foramen magnum to the 3rd lumbar vertebra. The spinal cord was removed and put in cold saline (0.9%). The adhering blood vessels were removed by pressing

Table 13 : Reagents and standards used for various estimations.

Sr. No.	Name	Method of preparation
1.	1-Amino-2-Naphthol-4-sulphonic acid reagent (ANSA) - I	To 195 ml of 15% sodium bisulphite were added 5 ml of 20% sodium sulphite and 0.5 g of ANSA. The contents were shaken and filtered through whatman No. 1 filter paper. The reagent was preserved at 4^{o} in a stoppered brown glass bottle.
2.	1-Amino-2-Naphthol-4-Sulphonic Acid reagent (ANSA) - II	To 10 ml of water, 19.25 mg of ANSA, 115.4 mg of sodium bisulphite and 115.4 mg. of sodium sulphite were added and shaken well and filtered through whatman No. 1 filter paper. This was stored at 4^{o} in a stoppered brown glass bottle.
3.	Bovine serum albumin (protein standard)	40 mg of bovine serum albumin was dissolved in 10 ml of water and from this 1 ml was diluted to 10 ml with water to give a final concentration of 400 mcg/ml.
4.	Brin Buffer	In 1000 ml beaker 20 ml of 0.9% sodium chloride, 515 ml of 1.15% KCl, 100 ml of 1.75% dipotassium hydrogen phosphate and 5 ml of 3.82% $MgSO_4.7H_2O$ were taken and mixed well. The pH of the solution was adjusted to 7.4 using 1N HCl.

* glass distilled water was used for preparing the reagents.

contd..

Table 13 : contd.

Sr. No.	Name	Method of preparation
5.	Cerebroside standard	10 mg of cerebrosides were dissolved in 10 ml of distilled alcohol, 1 ml of this was diluted to 10 ml with alcohol to give a final concentration of 100 mcg/ml.
6.	Chloroform:Methanol mixture (2:1)	Two volumes of chloroform were mixed with one volume of methanol. The mixture was shaken well and stored at 4^{0} in a dark bottle.
7.	Cholesterol standard	100 mg of cholesterol were dissolved in 100 ml of distilled alcohol. 10 ml of this was diluted to 50 ml with alcohol to give a concentration of 200 mcg/ml.
8.	30 mM 2',3'-cyclic adenosine monophosphate	118.8 mg of 2',3'-cyclic adenosine monophosphate were dissolved in 10 ml of water.
9.	1% copper sulphate	1g of copper sulphate were dissolved in 100 ml water.
10.	1 mM cytidine diphosphoryl (^{14}C) choline (^{14}C-CDP choline)	5.0 mg of cold CDP-choline were dissolved in 10 ml of water. To this a known amount (around 5 μci) of radioactive CDP-^{14}(C) choline was added in such a way that 20 ul of CDP-choline added

contd...

Table 13 : contd.

Sr. No.	Name	Method of preparation
		to the incubation medium would contain a minimum radioactivity of 10000 cpm. The concentration of CDP-choline was assayed by measuring the absorption of cytidine nucleotide at 260 and 280 nM in a spectrophotometer.
11.	1 mM cytidine diphosphoryl (^{14}C)-Ethanolamine (CDP-^{14}(C)-Ethanolamine)	This was prepared exactly the same way as CDP-choline using 4.6 mg of CDP-ethanolamine.
12.	1 mM Dicaprin	4.0 mg of dicaprin were taken in a vial and to this 10 ml of warm (60^{0}) 0.02 M Tris HCl buffer (pH 7.35) were added and shaken well on a vortex mixer. The contents of the vial was sonicated for 1 min. at 4^{0} using Biosonic IV sonicator (Bronwill, Rochester, U.S.A).
13.	1 mM Dithiothreitol (DTT)	15.4 mg of DTT were dissolved in 10 ml of water.
14.	10 mm EGTA	23 mg of EGTA were dissolved in 10 ml of water.
15.	0.5 N HCl	4.2 ml of concentrated (12N) HCl were diluted to 100 ml with water.

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Table 13 : contd.

Sr. No.	Name	Method of preparation
16.	0.2 M Imidazole buffer (pH 6.2)	1.36 g of imidazole were taken in 50 ml of water and the pH adjusted to 6.2 with IN HCl. The volume was made to 100 ml with water.
17.	Iodine reagent	38 mg of iodine were dissolved in 50 ml of 3% KI solution. From this 1 ml was diluted to 10 ml with 3% KI.
18.	Iron reagent	2.5 g of $FeCl_3.6H_2O$ were dissolved in 100 ml of 87% orthophosphoric acid. 8 ml of this were diluted to 100 ml with concentrated sulphuric acid (36N).
19.	Lowry's 'A' solution	2% sodium carbonate was prepared by dissolving 2g of sodium carbonate in 100 ml of 0.1N sodium hydroxide.
20.	Lowry's 'B' solution	1% copper sulphate and 2% sodium potassium tartrate were prepared separately and mixed in a ratio of 1:1 just before use.
21.	Lowry's 'C' solution	2 ml of Lowry's 'B' solution were mixed with 100 ml of Lowry's 'A' solution.

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Table 13 : contd.

Sr. No.	Name	Method of preparation
22.	Lowry's 'E' solution	A mixture consisting of 50 g of sodium tungstate, 12.5 g of sodium molybdate, 350 ml of water, 25 ml of85% phosphoric acid and 50 ml of concentrated hydrochloric acid (12N) were refluxed in a sand bath (150^0) for 18 h. The solution was cooled and to this solution 75 g of lithium sulphate, 25 ml of water and a few (5-6) drops of bromine solution were added. The mixture was then boiled for 15 minutes to remove excess bromine, cooled to room temperature (30^0) and diluted to one litre with water and filtered through whatman No. 1 filter paper. This stock solution was diluted freshly with two volumes ofwater before use.
23.	0.2 M Magnesium chloride	4.66 g of $MgCl_2.6H_2O$ were dissolved in 100 ml of water.
24.	40 mM Manganese chloride	0.792 g of $MnCl_2.4H_2O$ were dissolved in 100 ml of water.

contd...

Table 13 : contd.

Sr. No.	Name	Method of preparation
25.	Molybdate I (2.5%)	2.5 g of ammonium molybdate were dissolved in 100 ml of 5N sulphuric acid.
26.	Molybdate II (5%)	50 g of ammonium molybdate were dissolved in 200 ml of water. To this were added 300 ml of 10N sulphuric acid and diluted to 1000 ml with water.
27.	N-Acetyl neuraminic acid (NANA) standard	10 mg of NANA were dissolved in 10 ml of distilled water. 1 ml of this was diluted to 10 ml with water to give a concentration of 100 mcg/ml.
28.	Orcinol reagent	100 mg of orcinol were dissolved in 100 ml of 25N sulphuric acid.
29.	0.88% potassium chloride (KCl)	0.88 g of KCl were dissolved in 100 ml of water.
30.	Phosphorus standard	87.87 mg of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 100 ml of water. 1 ml of this was diluted to 10 ml with water to give a concentration of 20 mcg of phosphorus/ml.
31.	3% Potassium iodide (KI)	3 g of KI were dissolved in 100 ml of water.

contd..

Table 13 : contd.

Sr. No.	Name	Method of preparation
32.	40 mM Ribose-5-phosphate	146.2mg of ribose-5-phosphate were dissolved in 10 ml of water.
33.	Scintillator	400 mg of BBOT were dissolved in 100 ml of toluene.
34.	Sedoheptulose-7-phosphate standard	5 mg of sedoheptulose-7-phosphate were dissolved in 5 ml of water. From this, 1 ml was diluted to 10 ml with water to give a concentration of 100 mcg/ml.
35.	2% Sodium arsenite	2 g of sodium arsenite were dissolved in 100 ml of 0.5N HCl
36.	0.01M Sodium carbonate	106 mg of anhydrous sodium carbonate were dissolved in 100 ml of water.
37.	0.2 M Sodium carbonate	2.12 g of anhydrous sodium carbonate were dissolved in 100 ml of water.
38.	25 mcM Sodium periodate	475 mg of sodium periodate were dissolved in 100 ml of 62 mM sulphuric acid.
39.	1% Sodium deoxycholate	100 mg of sodium deoxycholate were dissolved in 10 ml of 0.01 M Tris HCl buffer, pH 7.5.

contd...

Table 13 : contd.

Sr. No.	Name	Method of preparation
40.	2% Sodium potassium tartrate	2 g of sodium potassium tartrate were dissolved in 100 ml of water.
41.	0.32 M Sucrose	11 g of sucrose were dissolved in 100 ml of water.
42.	0.32 M Sucrose in 0.01M tris HCl buffer (pH 7.4)	11 g of sucrose were dissolved in 100 ml of 0.02 M tris HCl buffer (pH 7.4).
43.	1N Sulphuric acid	10 ml of concentrated sulphuric acid (36N) were diluted to 360 ml with water.
44.	25 N Sulphuric acid	150 ml of concentrated sulphuric acid (36N) were diluted to 220 ml with water.
45.	62 mM Sulphuric acid	0.35 ml of concentrated sulphuric acid (36N) were diluted to 100 ml with water.
46.	Thiamine pyrophosphate (TPP)	10 mg of TPP were dissolved in 10 ml of 'Brin' buffer.
47.	6% Thiobarbituric acid (TBA)	6 g of TBA were dissolved in water and the pH adjusted to 9.0 using 1N NaOH and the volume made upto 100 ml with water.

contd...

Table 13 : contd.

Sr. No.	Name	Method of preparation
48.	7% Trichloroacetic acid (TCA)	7 g of TCA were dissolved in 100 ml of water.
49.	7.50% Trichloroacetic acid (TCA)	7.5 g of TCA were dissolved in 100 ml of water.
50.	0.2 M Tris-HCl buffer, pH 7.5	0.605 g of tris (hydroxy methyl) aminomethane were dissolved in 50 ml of water and the pH adjusted to 7.5 with 1N HCl. The volume was made to 100 ml with water.
51.	0.2 M Tris HCl buffer, pH 7.6	0.605 g of tris (hydroxy methyl aminoethane) were dissolved in 50 ml of water and the pH adjusted to 7.6 with 1N HCl. The volume was made to 100 ml with water.
52.	Upper phase reagent	3 ml of chloroform, 47 ml of methanol and 48 ml of water were mixed well and stored at 4^{o} in brown bottle.

the tissue between folds of filter paper and the tissue was weighed.

Separation of gray and white matter from the brain :

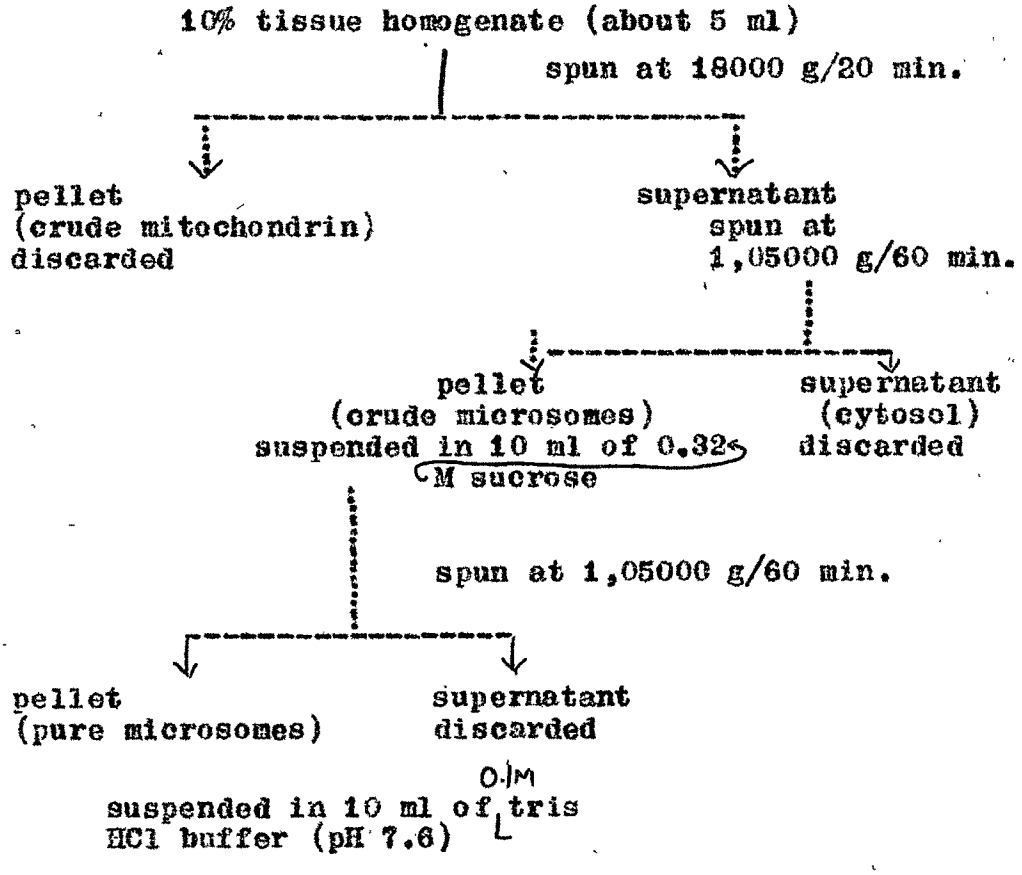
The pre-weighed brain was placed on a precooled glass plate kept on ice and the cerebellum and olfactory lobes removed. The gray portion of the cerebral cortex was removed using a sharp blade taking care to avoid any contamination of white material. The adhering blood vessels and water were removed by pressing the tissue between the fold of filter paper and weighed. This was used as 'gray matter'. The remaining parts of cerebral hemispheres were cut open and the white matter collected using a fine forceps and a needle. The brain stem was cut open and the gray matter seen was removed and the remaining white matter was collected. The white matter collected from the cerebral hemispheres and brain stem was pooled and surface moisture removed using the filter paper.

Preparation of microsomes :

The method of Radominska Pyrek and Horrocks (1972) as modified by Radominska pyrek et al (1977) was used for the preparation of microsomes. A 10% homogenate (about 500 mg of tissue was used) of the tissue was prepared using 0.32 M sucrose in 0.01 M tris HCl buffer (ph 7.4) and homogenizing the tissue at 4^{0} in a potter Elvehjem homogenizer at 1500 rpm.

It was homogenized first for 30 seconds with 6 up and down strokes. After an interval of 30 seconds the tissue was again homogenized for 30 seconds to achieve complete homogenization. The procedure followed to isolate microsomes from the homogenate is given in table. 14. The microsomal fraction obtained was dispersed in 10 ml of 1 M tris HCl buffer (pH 7.6) and diluted ten times with water and used for protein and enzyme assays.

Table 14 : Procedure for the isolation of microsomes.
(All the operations were carried out at 4°)



Biochemical estimations

Lipid extraction : The method of Folch et al (1957) modified by Suzuki (1965a) was used to extract lipids from the tissue. A known amount of tissue (roughly 1.5 g in the case of whole brain and 0.2 g in the case of gray matter, white matter or spinal cord) was ground with 20 volumes of cold chloroform: methanol mixture (2:1) ~~suspended in 10 ml of tris HCl buffer (7.6)~~ at room temperature (30^{o}) for 3-4 minutes using a glass mortar and pestle. The contents were filtered through whatman No. 1 filter paper and the residue on the filter paper immersed in chloroform:methanol mixture (2:1) along with the filter paper was kept overnight at 4^{o}. The residue from the filter paper was reextracted with 10 volumes of solvent mixture (the solvent used for overnight immersion was used and fresh solvent mixture added if necessary) and filtered again using whatman No. 1 filter paper. The filtrates were pooled together and the volume made upto 30 ml with solvent mixture in the case of brain, and 10 ml. in the case of gray matter, white matter or spinal cord. The combined extract was taken x in a test tube and shaken well with 0.2 volumes of 0.88% KCl solution. The contents were centrifuged in a clinical centrifuge at 3000 rpm for 5 min. The upper phase was collected using a pasteur pipette and the lower phase was treated with 0.2 volumes of upper phase reagent (chloroform

methanol : 0.88% KCl in the ratio of 3:47:48). The tube was again centrifuged and the upper phase was removed. The lower phase was again treated with 0.2 volumes of upper phase reagent, centrifuged and the upper phase was collected. All the upper phases were pooled together and dialysed at 4^{0} for 24 h against distilled water and the volume made to 10 ml with water. From this aliquots were used for the estimation of gangliosides. The lower phase (called the lipid extract) was diluted to 10 ml with chloroform:methanol mixture (2:1) and used for the estimation of cholesterol, phospholipids, galactolipids, plasmalogens and different classes of phospholipids.

Estimation of cholesterol

The method of Bowman and Wolf (1962) was used for the estimation of cholesterol. Aliquots (0.1-0.2 ml) of the lipid extract were taken in duplicates in test tubes and evaporated to dryness at 60^{0} in a water bath. To this 3 ml of distilled ethyl alcohol were added. Three ml of 'Iron reagent' were then added while shaking the contents of the tube on a vortex shaker. The color developed was read after 30 min at 540 nm in a Beckman spectrophotometer against a reagent blank which contained ethyl alcohol instead of the sample. A standard graph was prepared taking different concentrations of cholesterol (20-120 mcg) and treating them similarly.

Estimation of galactolipids

The method of Svennerholm (1956) modified by Neskovic et al (1972) was used for the estimation of galactolipids. Aliquots of lipid extract (0.1-0.4 ml) were taken in test tubes and evaporated to dryness at 60° in a water bath. To each tube 0.3 ml of ethanol was added and the tubes were again kept in 60° water bath for 3 min to dissolve the lipid in alcohol. After cooling to room temperature (30°), three ml of orcinol reagent were added. The tubes were shaken well on the vortex mixer and kept in a water bath at 80° for 20 min. After cooling under tap water and mixing their contents well on a vortex mixer, the color formed in the tubes were read against the reagent blank, which contained 0.3 ml. of alcohol instead of the lipid extract, at 505 nm in a Beckman spectrophotometer. A standard graph was prepared using different concentrations of standard cerebrosides (20-120 mcg).

Phospholipid estimation

Lipid phosphorus was estimated by the method of Bartlett (1959). Aliquots of 0.1-0.2 ml of the lipid extract were taken in test tubes in duplicate and evaporated to dryness at 60° in a water bath. One ml of 60% perchloric acid was added to each tube along with a small piece of porcelain. The tubes were heated for 30 min in a sand bath maintained at 230°. After the completion of digestion, as judged by the colorless appearance

of the solution, the tubes were cooled to room temperature (30^{0}) and volume made up to 1 ml with perchloric acid. To this was added 8.1 ml of distilled water, 0.5 ml of molybdate II reagent and 0.4 ml of ANSA-I reagent. The tubes were shaken well and kept in a boiling water bath for 8 min. They were then cooled at room temperature (30^{0}) and the color obtained was read against a reagent blank, containing only perchloric acid at 820 nm in a Beckman spectrophotometer. A standard graph was prepared by taking different concentrations of phosphorus standard (1-8 mcg). The values obtained for phosphorus were multiplied by 25 to obtain the phospholipid content of the sample.

Separation and estimation of various phospholipids

The separation of different phospholipids from the lipid extract was achieved using the thin layer chromatographic method of Horrocks (1968). Glass plates (20 x 20 cm) were cleaned in glass distilled water and coated to a thickness of 0.5 mm with silica gel 'G' (a slurry was prepared by taking 35 g of silica gel 'G' in 67 ml of 0.01 M Na_2CO_3. This can be used to coat 5 plates). The coated plates were air dried at room temperature (It takes 60 min to dry) and then activated for one hour at 110^{0} in an oven before use. The lipid extract, containing 10-20 mcg phosphorus, was spotted just one inch above the bottom of the plate. The plates were developed to a

height of 13-15 cm in a chamber presaturated for 60 min with chloroform : methanol : ammonia (65:25:4) solvent mixture. It took 60 min to develop. The plates were removed and air dried. The spots of different phospholipids were visualized by exposing the plates to iodine vapour.

For the separation of alkenylacyl glycerophosphoryl ethanolamine (Ethanolamine plasmalogens) from diacyl GPE the method of Horrocks and Sun (1972) was used. The lipid extract containing 10-20 mcg phosphorus was spotted at the lower left corner of silica gel plate. The plate was first developed to a height of 13-15 cm in chloroform:methanol :ammonia (65:25:4) solvent mixture. It was air dried for 10 min and exposed to concentrated hydrochloric acid fumes for 5 min and again air dried for 10 min. The second TLC run was made at right angles to the first in a solvent system containing chloroform:acetone: methanol:water (75:30:15:7.5) and allowed to run to a height of 10-12 cm. After this the plate was removed and air dried for 10 min and the spots were visualized by exposing the plate to iodine vapour.

The various phospholipid spots were detected using the appropriate authentic standards and scraped into the test tubes and the phosphorus was estimated by the method of Bartlett (1959). For blanks silica gel from unspotted area corresponding to the spot obtained was scraped and processed.

Estimation of plasmalogens

The method of Gottfried et al (1962) was used for the estimation of plasmalogens. Aliquots of lipid extract (0.2-0.4 ml) were taken in test tubes and evaporated to dryness in a 60^0 water bath. To the dried sample was added 0.5 ml of methanol and the same kept in a 60^0 water bath for 3 min. 0.5 ml of iodine reagent was then added and mixed well on a vortex mixer. After 10 min 4 ml of alcohol were added and again mixed well. The left over iodine was measured at 355 nm in a Beckman spectrophotometer against alcohol blank. 0.5 ml of iodine reagent was used as a standard instead of lipid extract and processed.

Estimation of gangliosides

Gangliosides in terms of N-acetylneuraminic acid moiety present were estimated by the method of Warren (1959) modified by Skoza and Mohos (1976). Aliquots of the upper phase obtained during lipid extraction (0.2-0.5 ml) were taken in test tubes in duplicate and the volume made upto 0.9 ml with glass distilled water. 0.1 ml of 1N H_2SO_4 was added and the sample digested in a water bath at 80^0 for 2 h to release the lipid bound NANA. The tubes were cooled to room temperature (30^0) and 0.25 ml of 0.25 mcM sodium periodate was added. After 30 min the excess of periodate was removed by adding 0.25 ml of 2% sodium arsenite and shaking well on a vortex mixer.

This was followed by the addition of 0.5 ml of thiobarbituric acid reagent. The tubes were kept in a boiling water bath for 7.5 min and then cooled to room temperature (30^{o}) and 2.5 ml of dimethyl sulfoxide was added. The readings were taken at 532 nm and 549 nm in a Beckman spectrophotometer against a blank containing 0.1N H_2SO_4 instead of the sample. The standard graph was prepared by taking different concentrations of NANA (5-30 mcg) and processing them as above. The interference due to other carbohydrate compounds in this estimation was corrected by using the following equation :

$$\text{Amount of NANA} = 0.09 \times \text{OD at } 549 - 0.03 \times \text{OD at } 532.$$

Estimation of protein

The method of Lowry et al (1951) was used for the estimation of protein. 0.1-0.2 ml of diluted tissue homogenate or microsomal fraction was taken in a test tube and the volume made to 0.6 ml with glass distilled water. To this was added 3.0 ml of Lowry 'C' reagent and allowed to stand for 10 min at room temperature (30^{o}). After this, 0.3 ml of Lowry 'E' was added and the contents in the tube were mixed well on a vortex mixer. The color developed in the next 30 min was read at 750 nm in Beckman spectrophotometer against a blank containing water instead of sample. Different concentrations (20-120 mcg) of standard protein (bovine serum albumin) were taken and processed as above to prepare a standard graph.

Assay system used for 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP)

The two step procedure of Kurihara and Takahashi (1973) was used for the assay of CNP activity. In this the first step involves the decyclization of the substrate (cyclic 2',3'-AMP) by the enzyme CNP. In the second step the dephosphorylation of the product formed (2'-AMP) is achieved by using pure alkaline phosphatase. The liberated inorganic phosphorus was estimated. The details of the procedure are indicated below :

I Step :

Activation of enzyme protein

Buffer	Tris HCl (pH 7.5), 0.2 M, 0.1 ml.
Detergent	Sodium deoxycholate 1%, 0.2 ml.
Enzyme (0.1 mg protein/ml)	0.2 ml
Conditions of incubation	25°, 5 min.

(b) Preincubation :

Buffer	Imidazole buffer (pH 6.2), 0.2 M, 0.1 ml.
Activated enzyme protein	0.2 ml (10-20 mcg)
Conditions of incubation	37°, 3 min.

(c) Incubation

Amount of substrate added to preincubated mixture	30 mM cyclic AMP, 0.1 ml.

Conditions of incubation	37^{o}, 20 min.
Termination of reaction	Addition of 0.2 ml of 0.2 M Na_2CO_3

(d) Treatment of blank

1. Water instead of enzyme in step b.
2. Water instead of substrate in step c.
3. Enzyme added after the addition of 0.2 M Na_2CO_3.

II Step

(a) Release of inorganic phosphorus

Aliquot taken from the incubated mixture	0.3 ml
Alkaline phosphatase	0.1 ml containing 50 mcg of enzyme protein.
Conditions of incubation	37^{o}, 1 h
Termination of the reaction	1 ml of 7% TCA was added and centrifuged.

(b) Estimation of inorganic phosphorus

For estimating inorganic phosphorus, 0.5 ml of the supernatant was taken and to it 0.05 ml of 2.5% ammonium molybdate I and 0.45 ml of ANSA-II reagent were added. Color developed in 10 min. was read at 720 nm in a Beckman spectrophotometer against a blank containing distilled water instead of supernatant.

(c) Enzyme unit	The amount of enzyme required to liberate 1 µmole of inorganic phosphorus under the assay conditions.
(d) Specific activity	Enzyme units/mg protein in enzyme preparation.

Assay systems used for 1,2-Diglycerol:CDP-choline phosphocholine transferase (PCT, EC 2.7.8.2) and 1,2-Diglycerol-CDP-ethanolamine phosphoethanolamine transferase (PET, EC 2.7.8.1).

Details of assay system and prodedure	Enzyme	
	PCT	PET
Basis of the method used	Radominska pyrek et al (1977)	Radominska pyrek et al (1977)
Tris HCl buffer @PH(7.6), 0.2 M	40 µl	40 µl
10 mM EGTA	20 µl	20 µl
1 mM DTT	20 µl	20 µl
0.2 M $MgCl_2$	20 µl	-
40 mM $MnCl_2$	-	20 µl
1 mM Dicaprin	20 µl	20 µl
1 mM CDP (^{14}C)-choline	20 µl	-
1 mM CDP (^{14}C)-Ethanolamine	-	20 µl
Start of reaction by adding microsomal enzyme protein (0.3 mg/ml)	20 µl	20 µl
Treatment of blank	Enzyme protein omitted	Enzyme protein omitted
Conditions of incubation	37^0, 20 min.	37^0, 20 min.
Termination of reaction	3 ml of 2:1 chloroform:methanol added and shaken well.	3 ml of 2:1 chloroform methanol added and shaken well.

Extraction and estimation of the product formed	The tube was centrifuged and the upper phase siphoned out with pasteur pipette. The lower phase was treated twice with 0.6 ml of chloroform:methanol:water (3:47:48) mixture and the upper phase removed by centrifugation. The lower phase containing the product (CPG or EPG) was transferred to radioactive vial. The tube was rinsed with chloroform: methanol (2:1) and the washings transferred to the vial. The contents in the vial evaporated by keeping the vial at 60° in an incubator. After cooling the sample to room temperature (25°), 10 ml of scintillator was added to the vial and the radioactivity of the product was measured in Beckman LSC-100.
Enzyme unit	Amount of enzyme required for the conversion of 1 nmole of substrate into product in an hour under the assay conditions.
Specific activity	Enzyme units per mg protein of enzyme preparation.

Assay system used for transketolase

The method of Dreyfus and Moniz (1962) was used for the assay of transketolase activity. The details of the assay conditions are given below :

(a)	Activation of the enzyme	
	Brin buffer	0.15 ml
	TPP solution	0.15 ml
	Enzyme (10% tissue homogenate)	0.2 ml
	Conditions of incubation	37°, 30 min.
(b)	Incubation	
	Substrate	0.1 ml of 40 mM ribose-5-phosphate
	Conditions of incubation	37°, 30 min.
	Termination of reaction	Addition of 1.4 ml of 7.5% TCA.
(c)	Treatment of blank	Addition of TCA before adding the substrate.
(d)	Estimation of the product formed (sedoheptulose 7-phosphate)	The samples was centrifuged and 1 ml of supernatant taken for the estimation of product. 4.5 ml of a mixture of cold sulphuric acid (36N) and water (5:1) were added to the sample and heated in a boiling water bath for 4 min, cooled and the color developed by adding

		0.1 ml of 3% cystein HCl and mixing thoroughly on a vortex mixer. The complete color was allowed to develop by keeping the tube for 5 h at room temperature (30^{0}). The colour developed was read at 520 nM against a reagent blank.
(e)	Reagent blank	1 ml of distilled water used instead of incubation mixture and processed as above.
(f)	Standard	Different concentrations (25-100 mcg) of sedoheptulose 7-phosphate were taken and processed as above.
(g)	Enzyme unit	Amount of enzyme required for the formation of 1 mcmole of sedoheptulose 7-phosphate per min under the assay conditions.
(h)	Specific activity	Enzyme units per mg protein of enzyme preparation.