MATERIALS AND METHODS

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The aim of the present investigations was to study the development of PolyPI in rat brain and the effect on the same of protein undernutrition induced during different periods of development. For studies on development the whole brain was used. For studies on the effects of undernutrition, whole brain as well as brain regions such as cerebral cortex, cerebellum and stem were used. PolyPI levels in the kidney were also estimated for possible differential effects of nutritional modifications in non-neural tissues.

The following parameters were employed :

(1) Body weight and weights of whole brain, cerebellum, brain stem and kidney.

(2) Concentrations of PtdIns(4,5)P_2 and PtdIns4P in whole brain, cerebral cortex, cerebellum, brain stem and kidney (at 0 and 1 min post-mortem in whole brain and at 0.75-1 min and 10 min post-mortem in brain regions and kidney).

(3) Concentrations of cerebrosides (total, NFA and HFA) in cerebral cortex, brain stem and cerebellum.

(4) Incorporation of $^{32}P_i$ in PtdIns(4,5)P_2 and PtdIns4P in the whole brain.

Studies on whole brain were carried out in this laboratory and studies on brain regions and kidney in Prof. G. Hauser's Laboratory at Harvard Medical School (U.S.A.) as the candidate

procured a fellowship to work with Prof. G. Hauser as a part of a collaborative research programme between Prof. C.V. Ramakrishnan and Prof. G. Hauser. Rats of Charles-Foster strain bred and maintained in the departmental rat colony were used for studies on whole brain. Sprague-Dawley rats obtained from Charles River Labs, Wilmington, MASS. were used for studies on brain regions and kidney.

EXPERIMENT - I

Ia. Changes in the concentration of PolyPI in rat brain at different times post-mortem

For this preliminary study rats of two ages (21 and 56 days old) were used and post-mortem losses in the levels of PtdIns(4,5)P_2 and PtdIns4P with time were determined. The heads of the animals were immersed in liquid N_2 at different times after decapitation viz. 2 sec, 1, 10, 20, 30, 40 and 70 min. The frozen heads were taken cut from liquid N_2, allowed to thaw partially in cold surroundings, the brains removed taking utmost care to maintain as low as temperatures as possible and then used for extraction and estimation of PolyPI. The extraction was carried out on the same day, samples stored at 4^{o} and then analysed within a fortnight.

Ib. Changes in the concentration of PolyPI pools in rat brain during development

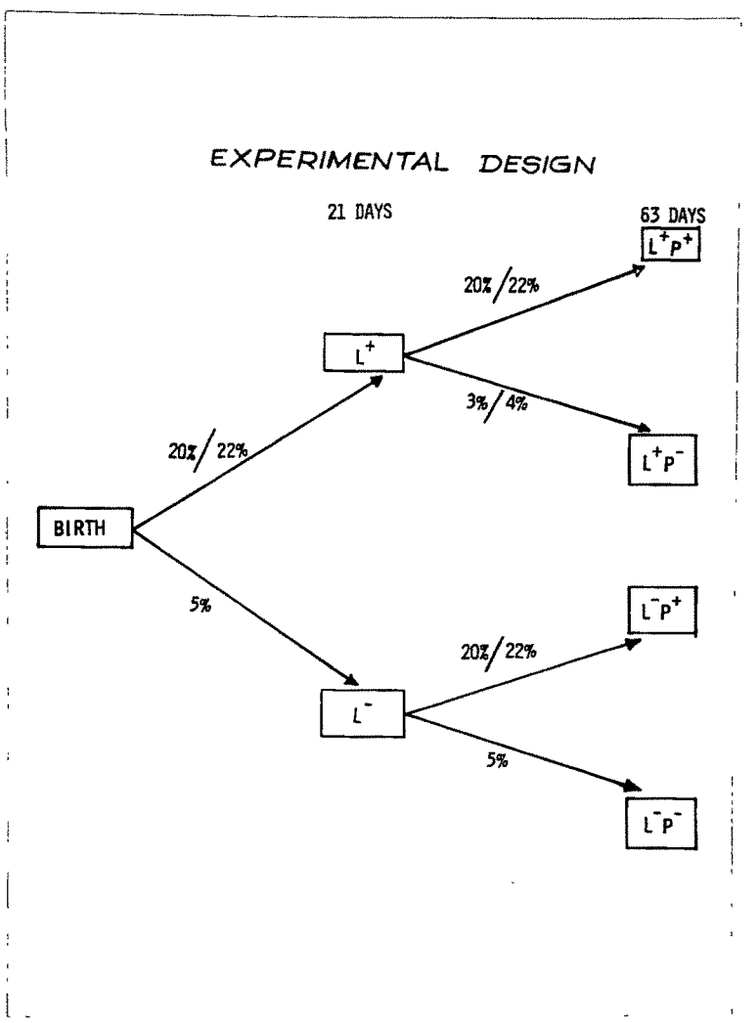
For this study rats of 0, 7, 14, 21, 34 and 63 days of age were used. After noting their body weights the heads of one set of rats were frozen in liquid N_2 immediately after decapitation and those of the second set frozen one minute later. These two time points were chosen based on the results of the preliminary study, Ia. The rest of the procedure remained the same as detailed in Ia.

EXPERIMENT - II

Effects of nutritional alterations during pre- and post-weaning periods on PolyPI pools in rat brain

The animal paradigm used for this study is shown diagrammatically in Fig. 9 and the composition of the diets is given in Table 20. Female rats weighing 200-250 g were used for breeding and during gestation they were fed a stock diet containing 18% protein. After delivery the litter size was adjusted to eight and the dams were fed 5% (L^-) or 20% (L^+) protein diets from birth to weaning and the pups used at this age for pre-weaning experiments. For post-weaning experiments L^- pups were continued on 5% (L^-P^-) or switched to 20% (L^-P^+) and L^+ pups continued on 20% (L^+P^+) or switched to 4% (L^+P^-) protein diets for a 6-week period. All weanling pups were individually caged and water was given ad lib for post-weaning

Fig. 9 : Experimental design.



Percentages refer to protein content of the diet.
L = lactation; P = post-weaning; + = animals were fed a diet containing 20-22% protein; - = animals were fed a diet containing 3-5% protein. The composition of the diets are given in Tables 20 and 21.

experiments. At 21 (L^+ and L^- groups) or 63 days (L^+P^+, L^+P^-, L^-P^+ and L^-P^- groups) rats were decapitated after noting their body weights and the heads frozen in liquid N_2 either immediately ("0 min" samples) or 1 minute later (1 min samples). The rest of the procedure remained the same as detailed in experiment I.

EXPERIMENT - III

Effects of pre- and post-weaning protein undernutrition on the incorporation of labelled $^{32}P_i$ in PolyPI of rat brain

For this study preliminary experiments were carried out to determine the peak time of incorporation of $^{32}P_i$ in PolyPI at 3 and 9 weeks of age. The animals were injected with 200 µci/100 g body weight of $^{32}P_i$ intraperitoneally. Weanling rats were decapitated at 0.25, 0.50, 1, 2, 3, 6, 8, 12, 18 and 24 hr and the 9-week-old rats at 0.50, 1, 2, 3, 4, 5, 6, 8 and 12 hr, after the injedtion. Liquid N_2 was not used for these preliminary experiments. The brains were removed and PolyPI extracted. The samples from thin layer chromatograms were taken in the scintillation fluid and counted.

The animal paradigm used for the main study was as described in the previous experiment - II. At 3 (L^+ and L^- groups) and 9 weeks of age (L^+P^+ and L^+P^- groups) rats were injected with 200 µci/100 g body weight of $^{32}P_i$ and decapitated at the peak time of incorporation of $^{32}P_i$ in PolyPI determined in the preliminary experiments viz. -- 4 and 2 hr after the

injection in 3- and 9-week-old rats, respectively. After decapitation the heads of the animals were frozen in liquid N_2 either immediately ("0 min" samples) or 1 minute later (1 min samples). The rest of the procedure remained the same as detailed in experiment I except that the samples from thin layer chromatograms were taken in the scintillation fluid and counted.

EXPERIMENT - IV

Effects of nutritional alterations during pre- and post-weaning periods on PolyPI pools in rat brain regions

The animal paradigm used for this study was the same as described in experiment II with minor modifications in the composition of the diets used. The design is shown diagrammatically in Fig. 9 and the composition of the diets is given in Table 21. Pregnant female rats were obtained from commercial sources and fed the 22% protein diet until delivery. After delivery the litter size was adjusted to eight and the dams were fed 5% (L^-) or 22% (L^+) protein diets from birth to weaning and the pups used at this age for pre-weaning experiments. For post-weaning experiments L^- pups were switched to 22% (L^-P^+) and L^+ pups were given 22% (L^+P^+) or 3% (L^+P^-) protein diets, for a 6-week period. All weanling pups were individually caged and water was given ad lib for post-weaning experiments. At 21 (L^+ and L^- groups) or 63 days (L^+P^+, L^+P^- and L^-P^+ groups) rats were decapitated after noting their body

weights and the dissection was begun either immediately ("0 min" samples) or 10 minutes later (10 min samples). The frozen brain regions were quickly weighed and used for extraction and estimation of PolyPI. The extraction was carried out on the same day, samples stored at 4^{o} and analysed within a fortnight.

EXPERIMENT - V

Effects of nutritional alterations during pre- and post-weaning periods on PolyPI pools in rat kidney

For this study animals from experiment IV were used. At 21 (L^{+} and L^{-} groups) or 63 ($L^{+}P^{+}$, $L^{+}P^{-}$ and $L^{-}P^{+}$ groups) days rats were decapitated after noting their body weights and the kidneys removed and frozen in liquid N_2 either immediately ("0 min" samples) or 10 minutes later (10 min samples). The frozen kidneys were weighed quickly and used for extraction and estimation of PolyPI. The extraction was carried out on the same day, samples stored at 4^{o} and analysed within a fortnight. **The details of all these experiments are summarized in Table 25.**

DIETS

For Baroda experiments, animals were fed either stock diet or experimental diets prepared in the laboratory. For Harvard experiments, they were purchased from commercial sources. The composition of these diets are given in Tables 19, 20 and 21.

Casein from buffalo milk, used for Baroda experiments was obtained from Amul Milk Dairy, Anand. It was washed first with

TABLE 19 : COMPOSITION OF STOCK DIET USED FOR EXPERIMENT - I.

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 catiang)	80
Groundnut oil (Peanut oil)	70
Fenugreek leaves (Trigonella foenum graceum)**	70
Protein content***	18%

* Flour sweepings from Amul Milk Dairy, Anand, containing skim milk powder (buffalo milk),

** Fenugreek leaves were used when available in season, otherwise vitamin A acetate in oil (5000 I.U. per kg of diet) was used.

*** The protein content was calculated by estimating total nitrogen and multiplying by 6.25.

TABLE 20 : COMPOSITION OF DIETS USED FOR EXPERIMENTS II AND III (BARODA).

		Dietary protein		
		4%[a]	5%[b]	20%
Washed casein (buffalo milk)	(g)	4.8	6.0	24.0
Vitamin mixture*	(g)	2.0	2.0	2.0
Salt mixture**	(g)	4.0	4.0	4.0
Groundnut oil	(g)	7.0	7.0	7.0
Sucrose	(g)	-	20.0	20.0
Sago (Metroxylon sago)	(g)	82.2	61.0	43.0

a Diet used during post-weaning period (L^+P^- group of animals).

b Diet used during lactation and post-weaning period (L^- and L^-P^- groups of animals).

* Composition given in Table 22.

** Composition given in Table 23.

The three diets were isocaloric and made available ad libitum.

TABLE 21 : COMPOSITION OF DIETS USED FOR EXPERIMENTS IV AND V (HARVARD).

		Dietary protein		
		3%[a]	5%[b]	22%
Washed casein (cow milk)	(g)	3.5	6.0	26.0
Vitamin mixture*	(g)	2.0	2.0	2.0
Salt mixture**	(g)	5.0	5.0	5.0
Cotton seed oil (hydrogenated)	(g)	8.0	8.0	8.0
Sucrose	(g)	-	18.0	8.7
Dextrin (vitamin free)	(g)	81.5	61.0	50.3
dl-methionine	(g)	0.15	0.15	0.15

a Diet used during post-weaning period (L^+P^- group of animals).

b Diet used during lactation (L^- group of animals).

* Composition given in Table 22.

** Composition given in Table 23.

The three diets were isocaloric and were made available ad libitum.

alcohol and then with tap water to remove the alcohol and finally with distilled water. The washed casein was dried and used. The nitrogen content of this casein was found to be 13.3/100 g. For Harvard experiments casein from cow milk was used and since this casein is known to be deficient in methionine, the control as well as the experimental diets were supplemented with methionine (0.15%).

Commercially available sago prepared from tapioca flour (Manihot utillissima) was ground and used as a starch source for Baroda experiments as it contains only 0.2% protein and 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 (Booher et al., 1951).

The vitamin mixture used for preparing diets for Baroda, experiments has been 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 22. The salt mixture used was the Hawk-Oser salt mixture, No. 3 (Hawk et al., 1954), the composition of which is given in Table 23. Both mixtures were prepared in bulk and stored in airtight bottles. The vitamin mixture was stored at 4^{o} in dark brown coloured glass bottles. The experimental diets were prepared once a week and the stock diet was prepared every day. Groundnut

TABLE 22 : COMPOSITION OF VITAMIN MIXTURES.

	Amount g/100g of mixture	
	Mixture used for Harvard experiments	Mixture used for Baroda experiments
Thiamine hydrochloride (mg)	30.0	20.0
Riboflavin (mg)	30.0	20.0
Pyridoxine hydrochloride (mg)	35.0	5.0
Niacin (mg)	100.0	75.0
Calcium-d-pantothenate (mg)	80.0	50.0
Choline chloride (mg)	1,500.0	3,750.0
Myo-inositol (mg)	1,000.0	1,000.0
Para amino benzoic acid (mg)	-	50.0
Folic acid (mg)	10.0	5.0
Menadione sodium bisulfite (mg)	0.25	-
d-biotin (mcg)	1,000	0.02
Cyanocobalamin (mcg)	50.0	25.0
Vitamin A acetate (IU)	20,000	25,000
Cholecalciferol (IU)	5,000	4,690
dl-alpha-tocopherol acetate (IU)	250	-
Sucrose	To make 100g of final mixture.	

TABLE 23 : COMPOSITION OF SALT MIXTURES.

	Amount g/100g of mixture	
	Mixture used for Harvard experiments	Mixture used for Baroda experiments
Calcium carbonate	28.49	6.85
Calcium hydrogen phosphate	14.80	11.28
Dicalcium tricitrate	-	30.82
Dipotassium hydrogen phosphate	20.50	21.87
Potassium chloride	-	12.47
Sodium chloride	9.50	7.70
Magnesium sulphate (anhydrous)	6.81	3.83
Magnesium carbonate	-	3.51
Sodium dihydrogen phosphate	11.80	-
Ferric ammonium citrate	-	1.53
Ferric citrate	0.72	-
Copper sulphate	0.12	0.10
Sodium fluoride	0.022	0.013
Manganese sulphate	0.40	0.018
Potassium aluminium sulphate	-	0.009
Potassium iodide	0.0015	0.004
Zinc chloride	0.085	-
Sodium solonate	0.001	-
Chromium chloride	0.031	-
Cobalt chloride	0.026	-
Dextrin	5.593	-

oil (peanut oil) was added at the time of feeding. The vitamin and salt mixtures used for experiments at Harvard were obtained from Ralston Purina test diets (U.S.A.). The compositions of the same are given in Tables 22 and 23.

CHEMICALS :

The chemicals and solvents used for all experiments were of research grade purity. For Baroda experiments they were obtained either from British Drug House or Sarabhai Chemicals, and for Harvard experiments from Fisher Scientific Company or Mallinckrodt Chemical Works. The following chemicals were obtained from the sources indicated against them.

Chemicals	Source
Cerebrosides	Sigma Chemical Co., U.S.A.
Diphenyl oxazole (PPO)	V.P. Chest Institute, Delhi India.
Diphenyl oxazolyl benzene (POPOP)	
Perchloric acid	E. Merck, Germany
Silica Gel "H"	Loba-Chemie Wien Fischamend, Austria
Silica 60 precoated plates	E. Merck, Germany
Sodium dihydrogen ortho-phosphate ($NaH_2{}^{32}PO_4$)	Bhabha Atomic Research Centre, Trombay, Bombay, India.

The details regarding the preparation of reagents used for different estimations are given in Table 24.

TABLE 24: REAGENTS AND STANDARDS USED FOR DIFFERENT ESTIMATIONS.

Sr. No.	Reagent	Method of preparation
1.	Alkaline CH_3OH	Four volumes of 80% CH_3OH were mixed with one volume of 1% sodium carbonate and shaken well.
2.	1-amino-2-naphthol-4-sulphonic acid (ANSA)	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 stored at 4^{o} in a stoppered brown glass bottle.
3.	Ammonium molybdate (1.25%)	1.25 g of ammonium molybdate were dissolved in 100 ml of glass distilled water (G.D.W.). It was heated over a low flame to dissolve the molybdate completely.
4.	Ammonium molybdate (5%)	50 g. of ammonium molybdate were dissolved in 200 ml of G.D.W. To this was added 300 ml of 10N sulphuric acid and diluted to 1000 ml with G.D.W.
5.	Aqueous CH_3OH (80%)	80 ml of pure CH_3OH was diluted to 100 ml with G.D.W. and shaken well.

contd...

TABLE 24 : contd.

Sr. No.	Reagent	Method of preparation
6.	Ascorbic acid (5%)	5 g of ascorbic acid was dissolved in 100 ml of G.D.W. The reagent was prepared fresh just before use.
7.	Benzoylation reagent	1 ml of benzoyl chloride was added to 9 ml of pyridine and mixed well using a vortex mixer. The reagent was prepared fresh just before use.
8.	Cerebroside standard	1.5 mg of cerebroside was dissolved in 3 ml of $CHCl_3$:CH_3OH (2:1 v/v) to give a standard concentration of 500 µg/ml.
9.	$CHCl_3$:CH_3OH mixture (2:1 v/v)	Two volumes of $CHCl_3$ were mixed with one volume of CH_3OH and shaken well.
10.	$CHCl_3$:CH_3OH mixture (1:1 v/v)	One volume of $CHCl_3$ was mixed with one volume of CH_3OH. To 100 ml of the mixture 66 mg of $CaCl_2$ were added and shaken well.
11.	$CHCl_3$:CH_3OH:1N HCl mixture (3:48:47 v/v/v)	3 volumes of $CHCl_3$, 48 volumes of CH_3OH and 47 volumes of 1N HCl were mixed and shaken well.
12.	$CHCl_3$:CH_3OH:0.01N HCl mixture (3:48:47: v/v/v)	3 volumes of $CHCl_3$, 48 volumes of CH_3OH and 47 volumes of 0.01N HCl were mixed and shaken well.

TABLE 24 : contd.

Sr. No.	Reagent	Methods of preparation
13.	Hydrochloric acid 1N	10 ml of conc. HCl was diluted to 120 ml with G.D.W.
14.	Phosphorus standard	87.87 mg of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 100 ml of G.D.W. 1ml of this stock was diluted to 10 ml with G.D.W. to give concentration of 20 µg of phosphorus/ml.
15.	Potassium chloride (0.88%)	880 mg of KCl were dissolved in 100 ml of G.D.W.
16.	Potassium oxalate (0.3 - 2%)	0.3 - 2 g of potassium oxalate were dissolved in 100 ml of G.D.W.
17.	Scintillator	400 mg of diphenyl oxazole (PPO) and 40 mg of diphenyl oxazolyl benzene (POPOP) were added to 100 ml of toluene. The solution was placed in a warm water bath for dissolving the material completely.
18.	Sodium carbonate (1%)	1g of sodium carbonate was dissolved in 100 ml of G.D.W.
19.	Sodium chloride (0.9%)	900 mg of sodium chloride were dissolved in 100 ml of G.D.W.

REMOVAL OF FROZEN BRAIN TISSUE

For experiments on whole brain the heads of one set of rats were frozen immediately in liquid N_2 after decapitation and in the second set they were frozen one min after decapitation. The time taken for the heads to freeze properly appeared to depend on the thickness of the skull. In one of the experiments they were frozen at 10, 20, 30, 40 and 70 min after decapitation. The frozen heads were removed from liquid N_2, allowed to thaw partially in cold surroundings (time taken varied from 1-3 min depending on the age of the animal), the brains removed, taking utmost care to maintain as low temperatures as possible, and then used for extraction and analyses of PolyPI.

DISSECTION OF BRAIN REGIONS AND KIDNEY

Dissection of brain regions, namely cerebral cortex, cerebellum and stem was done by visual inspection. Rats were decapitated and the dissection was begun either immediately ("0 min" samples) or after leaving at room temperature for 10 min (10 min samples). Kidneys and brains were removed simultaneously by two investigators as rapidly as possible and cerebellum and brainstem were dissected immediately. The rest of the brain was placed on a piece of whatman filter paper and dipped into liquid N_2 for 15-20 sec in order to halt the further degradation of PolyPI. Gray matter was sliced carefully

with a scalpel from the lateral surfaces of the cerebral hemispheres in a frozen state. This procedure took 50-60 sec. The elapsed time until freezing of cerebellum, brainstem and kidney and immersion of the cerebral hemispheres was approximately 60 sec.

All tissues were weighed quickly under frozen conditions and used for extraction of PolyPI immediately. Regions from 2 to 8 animals were pooled as required, to provide samples containing sufficient PolyPI for analyses.

EXTRACTION OF POLYPHOSPHOINOSITIDES

The method of Hauser and Eichberg (1973) was used for the extraction of PolyPI in Harvard experiments. For Baroda experiments this method was slightly altered to suit the conditions prevailing in this laboratory. The detailed method of extraction and the alterations made at different steps is given below :

One gram of the tissue sample was homogenized for 3 min with 15 volumes of $CHCl_3$:CH_3OH (1:1 v/v) containing 60 µmoles of $CaCl_2$. This homogenization was carried out in a virtis homogenizer for Harvard experiments and for Baroda experiments a pestle and mortar was used. After homogenization the extract was filtered through whatman No. 1 filter paper. The ratio of $CHCl_3$:CH_3OH was adjusted to 2:1 v/v by adding extra chloroform.

The residue was washed twice with three volumes of $CHCl_3$:CH_3OH (2:1 v/v). The combined filtrates were washed with 0.2 volumes of 0.88% KCl and the lower phase of the neutral solvent extract was used for the analysis of cerebrosides.

The damp tissue residue from the neutral solvent extraction step was used for acid extraction. For Harvard experiments each sample was extracted with 24 volumes of $CHCl_3$:CH_3OH (2:1 v/v) containing 0.25% HCl (12N). The extraction was carried out in three stages by adding 8 volumes of the solvent and incubating at 37° for 20 min each time. For Baroda experiments, a mechanical shaker was tried, to reduce the manual labour. When the samples were shaken for 1 hr at 150 r.p.m. with $CHCl_3$:CH_3OH (2:1 v/v) containing 0.25% HCl (12N) recoveries were low but on increasing the concentration of HCl to 0.5%, better recoveries were obtained. Hence the acid extraction was done by shaking the samples in $CHCl_3$:CH_3OH (2:1 v/v) containing 0.5% HCl (12N) in a mechanical shaker for one hr.

The acid extract was shaken with 0.2 volumes of 1N HCl. The lower phase was successively washed with two-thirds its volume of $CHCl_3$:CH_3OH:1N HCl (3:48:47 v/v/v) and $CHCl_3$:CH_3OH: 0.01N HCl (3:48:47 v/v/v). The washed lower phase was cleared with 4-5 drops of CH_3OH and adjusted to pH 7-8 by adding 1-2 drops of 15N NH_4OH. The extract was stored at 4° and used for analyses of PolyPI.

SEPARATION OF POLYPHOSPHOINOSITIDES

PtdIns(4,5)P_2 and PtdIns4P were separated by thin layer chromatography (TLC) according to the method described by Gonzalez-Sastre and Folch (1968). For Baroda experiments, TLC plates were prepared in the laboratory. Glass plates (20 cm x 20 cm) cleaned in G.D.W. were coated to a thickness of 0.30 mm with silica gel "H" containing 0.3-2% of potassium oxalate (30 g of silica gel was slurried in 60 ml of potassium oxalate solution for 5 plates). The concentrations of Ca^{2+} in the gel obtained from different firms were different, and there was variation from batch to batch in the gel supplied by the same firm. It was therefore necessary to determine the concentration of potassium oxalate required every time a new bottle of gel was used.

The coated plates were air dried and then activated for 30 min at 110° before use. Samples containing 3-10 µg of PolyPI phosphorus were evaporated in a warm water bath maintained at 60°. The evaporated samples were dissolved in minimum quantities of $CHCl_3$:CH_3OH:H_2O (7.5:2.5:0.2 v/v/v) solution and then applied to the activated plates quantitatively. Each sample was washed 3-4 times with approximately 6 drops of the solvent. Each plate was divided into four lanes of equal width and the samples were spotted as thin bands in the middle of each lane one inch above the bottom of the plate. The plates were developed at 30° to a height of 18 cm in a tank

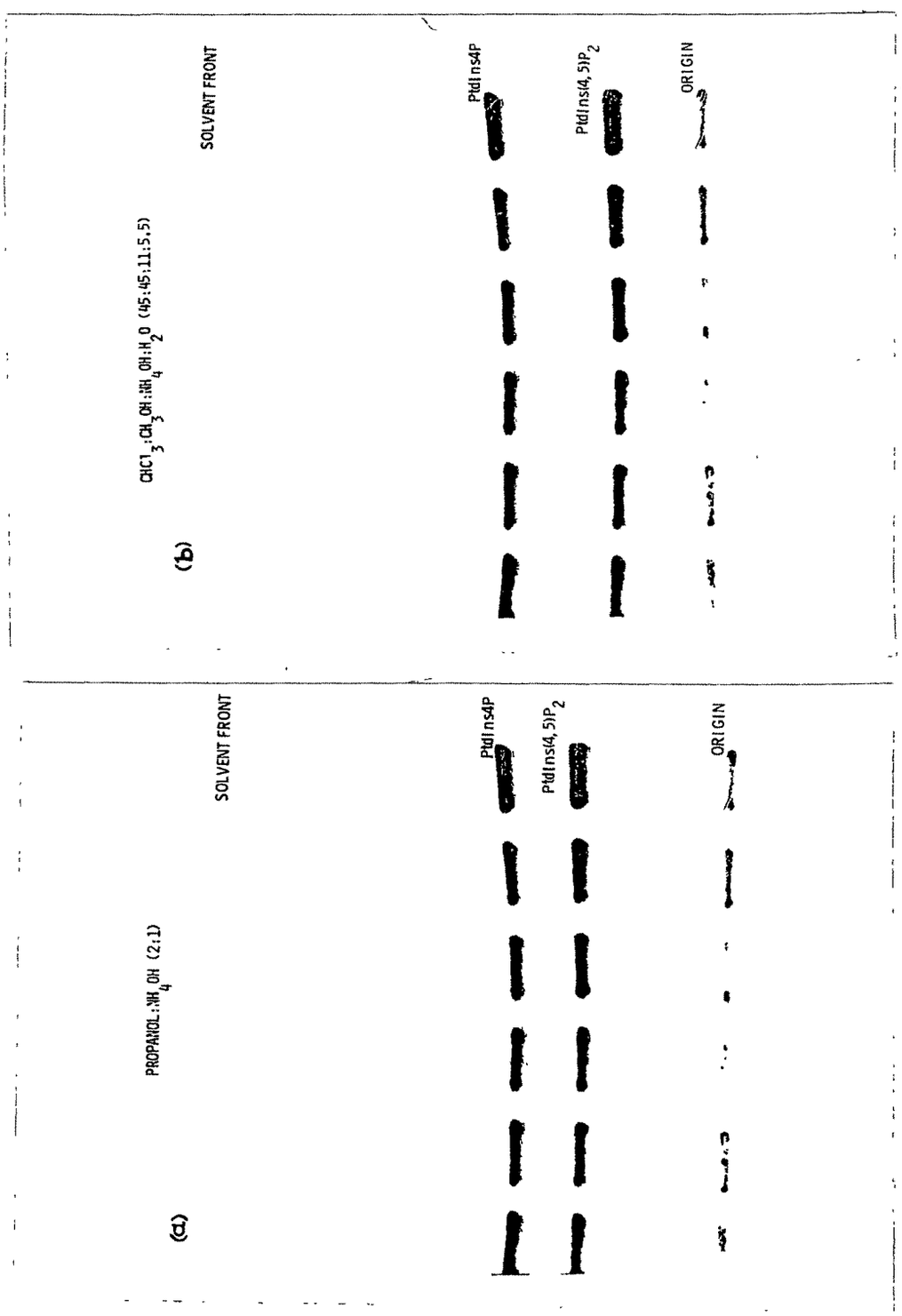


Fig. 10 : Thin layer chromatographic separation of PolyPI.

saturated for 45-60 min with a solvent system containing n-propanol and 4N NH_4OH in the ratio of 2:1 v/v. The time taken for the run ranged from 6-8 hr depending on the temperature and humidity of the surroundings. The spots were revealed by exposure to iodine vapours. The Rf value for PtdIns(4,5)P_2 was 0.29 and for PtdIns4P 0.43 (Fig. 10).

For Harvard experiments pre-coated silica 60 plates were used and the samples were spotted on the plates directly after evaporation under N_2 atmosphere in the same manner as described above. The plates were developed in a solvent system containing $CHCl_3$:CH_3OH:NH_4OH:H_2O (45:45:11:5.5 v/v/v/v) at 30° to a height of 18 cm (Pappu and Hauser, 1981). The time taken for the run ranged from 2-2$\frac{1}{2}$ hr. The spots were revealed by exposure to iodine vapours and the Rf value for PtdIns(4,5)P_2 was found to be 0.11 and for PtdIns4P 0.44 (Fig. 10). This solvent-system had two advantages over the n-propanol:4N NH_4OH solvent system -- the run was faster and PtdIns(4,5)P_2 and PtdIns4P were separated from each other by a greater distance as indicated by the Rf values thereby reducing the chances of contamination of one lipid by the other. All samples were run in duplicate and sometimes in triplicate if necessary.

BIOCHEMICAL ESTIMATIONS

Phosphorus

At Baroda PolyPI phosphorus was estimated by the method of Bartlett (1959). PtdIns(4,5)P_2 and PtdIns4P spots were

scraped from the TLC plates and placed into clean test tubes prewashed with acid and G.D.W. The inositide-bound phosphorus was liberated by adding one ml of 60% perchloric acid to each tube along with a small piece of porcelain and heating the tubes for half an hr in a sand bath maintained at 230°. The completion of digestion was judged by the colourless appearance of the solution. To this were added 3.1 ml of G.D.W., 0.5 ml of 5% ammonium molybdate solution and 0.4 ml of ANSA reagent. The tubes were shaken well and kept in a boiling water bath for 8 min. They were then cooled to 30° and the absorbance read at 820mm in a Beckman Spectrophotometer against a reagent blank containing all the reagents except the sample. Silica gel from the unspotted area was scraped and used as a gel blank. Blanks containing 80 mg of silica gel gave readings equivalent to 0.2-0.4 µg of phosphorus. A standard graph was prepared by using different concentrations of phosphorus (1-6 µg).

In Harvard a more sensitive method had to be used for phosphorus analysis since the cerebral cortex samples contained very low amounts of PolyPI phosphorus. The micro method of Houser et al (1966) was therefore employed. Digestion was carried out by adding 0.25 ml of 60% perchloric acid and heating for 20 min in a digestion chamber maintained at 230°. The tube was then cooled and to this was added 0.92 ml of G.D.W., 0.4 ml of ammonium molybdate (1.25%) and 0.4 ml of vitamin C (5%). The

tubes were shaken well and kept in boiling water bath for 5 min. After colour development they were centrifuged for 5 min and readings of the supernatant taken at 797 nm in a Beckman Spectrophotometer against a reagent blank containing all reagents except the sample. Blanks containing 80 mg of solica gel gave readings equivalent to 0.008 µg of phosphorus. A standard graph was prepared by using different concentrations of phosphorus (0.25-2 µg).

Cerebrosides:

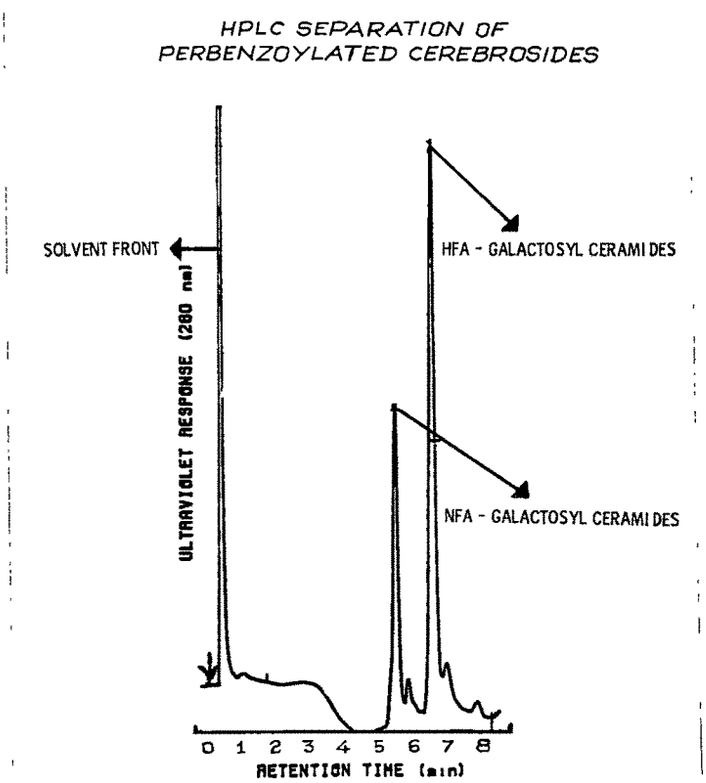
A gradient elution high performance liquid chromatography method (HPLC) with detection at 230 nm as described by Jungalwala et al (1977) was used for the analysis of perbenzoylated cerebrosides containing hydroxy and non-hydroxy fatty acids. The analysis was carried out on the neutral solvent extract (see page 157) after washing once with 0.2 volumes of 0.88% KCl. Samples containing 100-200 ug of cerebroside standards and appropriate amounts of lipid extracts from tissues were benzoylated.

All samples were placed in screw cap reactivials and the organic solvent evaporated. They were then made moisture-free by drying over P_2O_5 overnight in a vacuum desiccator. 250 µl of 10% (v/v) benzoyl chloride in dry pyridine was added to each sample. After heating for 1 hr at 60° in an oil bath, the pyridine was removed under a stream of N_2. To the dried samples

1.5 ml of alkaline-CH_3OH (4 volumes of CH_3OH and 1 volume of 1% aqueous Na_2CO_3) was added, followed by 2.5 ml of hexane. The vials were capped, vigorously shaken on a vortex mixer and briefly centrifuged to separate the two phases. Minor modifications were required because the total lipid extract contained material insoluble in hexane. To remove this material the hexane phases after the first alkaline washing was transferred into a new set of tubes and further washed thrice with 1.5 ml of alkaline CH_3OH, discarding the lower phase each time. The samples were then washed thrice with 1.5 ml of 80% aqueous CH_3OH in order to improve the separation of the phases. After the final wash they were evaporated under a stream of N_2. The benzoylated cerebrosides were redissolved in a small volume of CCl_4 (250 µl) for HPLC.

Suitable portions of the samples containing approximately 1-10 nmoles of cerebrosides were injected onto the Zipax column which was equilibrated with 23.8% dioxane in hexane. Elution was carried out with a 15 min linear gradient of 0.47% - 20% dioxane in hexane. The solvents were pumped at the rate of 2 ml per min. After completion of an analysis the column was regenerated by reversing the gradient and equilibrating the column with the original solvent mixture. Since a rise in the baseline was obtained with increasing amounts of dioxane in hexane, the instrument was programmed to correct

Fig. 11 : High performance liquid chromatographic separation of perbenzoylated cerebrosides.



1 µg of beef brain cerebroside standard was injected on a Zipax column. Elution was with a 15 min linear gradient of 0.47% - 20% dioxane in hexane pumped at the rate of 2 ml per min.

the baseline. The elution pattern for standard cerebrosides is given in Fig. 11.

HPLC analysis was performed with a Waters Associates (Milford, MA) Model 6000 solvent delivery system combined with a solvent programmer Model 660 and a Model U-6K injector. The chromatographic column was a 50 cm x 2.1 mm ID stainless steel tube packed with Zipax, pellicular particles, average size of 27 μm covered with porous silica (E.I. DuPont de Nemours, Inc., Wilmington, DE). Detection was done with a Schoeffel Instruments Corporation (Westwood, NJ) Model SF 770 variable-wavelength spectromonitor. This monitor had an 8 μl flow through the cells. The monitor was coupled to an Omniscribe TM Stripchart recorder. The peak areas were measured with an Autolab Minigrator (Spectra Physics, Santa Clara, CA). Cerebroside analysis was restricted to samples of cerebral cortex, cerebellum and brain stem for pre- and post-weaning experiments carried out at Harvard. Since cerebrosides are known to be myelin markers the analysis was done to check for white matter inclusion in the cerebral cortex samples so that a correction factor could be applied to obtain the actual concentration of PolyPI.

RADIOACTIVITY MEASUREMENTS

For radioactivity measurements using $^{32}P_i$ as tracer the bands (PtdIns(4,5)P_2 and PtdIns4P) were scraped and taken in

5 ml of scintillation fluid (0;4% diphenyl oxazole (PPO) and 0.04% diphenyl oxazolyl benzene (POPOP) in toluene). The samples were counted in a double-channel liquid scintillation counter.

TABLE 25 : EXPERIMENTAL DETAILS.

Expt. no.	Expt. title	Diets used for the animals or their mothers	No.of Groups	Period of treatment (days)	No. of rats used		Parameters measured
					Age/ group	No. used	
1	2	3	4	5	6	7	8
I a	Changes in the concentration of PolyPI in rat brain at different time periods post-mortem	stock diet (18% protein)	2	-	21 days 56 days	21 16	PtdIns(4,5)P_2 and PtdIns4P concentrations at 2 sec, 1, 10, 20,30, 40 and 70 min post-mortem
b	Changes in the concentration of PolyPI pools in rat brain during development	stock diet (18% protein)	6	-	0 7 14 21 34 63	16 32 13 18 14 8	PtdIns(4,5)P_2 and PtdIns4P,concentration at "0 min" and 1 min post-mortem
II a	Effect of pre-weaning under-nutrition on PolyPI pools in rat brain.	mothers fed high (20%) or low (5%) protein diet during lactation.	2 control (L^+) under-nourished (L^-)	from birth to 21 days of age	L^+ L^-	18 12	"

contd.

TABLE 25 : contd.

1	2	3	4	5	6	7	8
b	Effect of post-weaning protein deficiency on the concentration of PolyPI pools in rat brain.	Weanling rats fed high (20%) or a low (4%) protein diet during during post-weaning period for 6 weeks.	2 control (L^+P^+) undernourished (L^+P^-)	3 to 9 weeks of age	L^+P^+ L^+P^-	8 8	$PtdIns(4,5)P_2$ and PtdIns4P concentrations at "0 min" and 1 min post-mortem.
c	Effect of post-weaning nutritional rehabilitation on the concentrations of PolyPI in brains of rats undernourished prior to weaning.	weanling rats reared by the mothers fed either 5% or 20% protein diet during lactation were fed 20% protein diet for 6 weeks	control (L^+P^+) rehabilitated (L^-P^+)	3 to 9 weeks of age	L^+P^+ L^-P^+	8 8	"
d	Effects of pre-weaning and post-weaning protein deficiency on the concentration of PolyPI pools in rat brain.	weanling rats reared by mothers fed either 5% or 20% protein diet during lactation were continued on the same diet for 6 weeks.	2 control (L^+P^+) continued (L^-P^-)	3 to 9 weeks of age	L^+P^+ L^-P^-	8 11	"
III a	Time course of labeling of $^{32}P_i$ into PolyPI of rat brain	stock diet (18% protein)	2	-	21 days 63 days	10 10	Incorporation of $^{32}P_i$ into PtdIns-$(4,5)P_2$ and PtdIns4P at different times after injection of the label.

contd..

TABLE 25 : contd.

1		2	3	4	5	6	7	8
								0.25, 0.5, 1,2,4,6, 8,12,18 and 24 hr - 21 days. 0.5, 1,2,3,4,5,6,8, 12 and 24 hr - 63 days.
	b	Effect of pre-weaning under-nutrition on incorporation of $^{32}P_i$ into PolyPI of rat brain.	mothers fed high (20% or a low (5%) protein diet during lactation	2 control (L^+) under-nourished (L^-)	from birth to 21 days of age	L^+ L^-	6 6	Incorporation of $^{32}P_i$ in PtdIns(4,5)P_2 and PtdIns4P at 0 min and 1 min post-mortem.
	c	Effect of post-weaning protein deficiency on incorporation of $^{32}P_i$ into PolyPI of rat brain.	weanling rats fed the high (20%) or a low (4%) protein diet during post-weaning period for 6 weeks.	2 control (L^+P^+) under-nourished (L^+P^-)	3 to 9 weeks of age	L^+P^+ L^+P^-	8 8	"
IV	a	Effect of pre-weaning under-nutrition on concentration of PolyPI in rat brain cerebral cortex, stem and cerebellum.	mothers fed the high (22%) or a low (5%) protein diet during lactation	2 control (L^+) under-nourished (L^-)	from birth to 21 days of age	L^+ L^-	36 44	PtdIns(4,5)P_2 and PtdIns4P concentrations at "0 min" and 10 min post-mortem. Cerebrosides in all samples of cerebral cortex and selected samples of cerebellum and brainstem.

contd...

TABLE 25 : contd.

1	2	3	4	5	6	7	8
b	Effect of post-weaning protein deficiency on concentration of PolyPI pools in rat brain cerebral cortex, cerebellum and stem.	weanling rats fed high (22%) or a low (3%) ~~or~~ protein diet during post-weaning period for 6 weeks.	2 control (L^+P^+) undernourished (L^+P^-)	3 to 9 weeks of age	L^+P^+ L^+P^-	16 29	PtdIns(4,5)P_2 and PtdIns4P concentrations at "0 min" and 10 min post-mortem. Cerebrosides in all samples of cerebral cortex and selected samples of cerebellum and brain stem.
c	Effect of post-weaning nutritional rehabilitation on concentration of PolyPI pools in cerebral cortex, cerebellum and brainstem of rats undernourished prior to weaning	weanling rats reared by mothers fed either 5% or 22% protein diet during lactation were fed 22% protein diet for 6 weeks	2 control (L^+P^+) rehabilitated (L^-P^+)	3 to 9 weeks of age	L^+P^+ L^-P^+	16 16	"
V a	Effect of pre-weaning undernutrition on concentration of PolyPI pools in rat kidney.	mothers fed high (22%) or a low (5%) protein diet during lactation	2 control (L^+) undernourished (L^-)	from birth to 21 days of age	L^+ L^-	36 44	PtdIns(4,5)P_2 and PtdIns4P concentrations at "0 min" and 10 min post-mortem

contd..

TABLE 25 : contd.

1	2	3	4	5	6	7	8
b	Effects of post-weaning protein deficiency on concentration of PolyPI pools in rat kidney	weanling rats fed high (22%) or low (3%) protein diet during postweaning period for 6 weeks	2 control (L^+P^+) undernourished (L^+P^-)	3 to 9 weeks of age	L^+P^+ L^+P^-	16 29	PtdIns(4,5)P_2 and PtdIns4P concentrations at "0 min" and 10 min post-mortem
c	Effect of post-weaning nutritional rehabilitation on concentration of PolyPI pools in kidney of rats undernourished prior to weaning	weanling rats reared by mothers fed either 5% or 22% protein diet during lactation fed 22% protein diet for 6 weeks	2 control (L^+P^+) rehabilitated (L^-P^+)	3 to 9 weeks of age	L^+P^+ L^-P^+	8 16	"