

## Effect of feeding protein deficient diet on phospholipid turnover and protein kinase C mediated protein phosphorylation in rat brain

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Feeding of protein deficient diet is known to alter the transmembrane signalling in brain of rat by reducing total protein kinase C (PKC) activity. Phospholipid metabolism regulates the activation of PKC through generation of second messengers and the extent of PKC activation accordingly influences the magnitude of phosphorylation of its endogenous substrate proteins. Thus it was speculated that ingestion of protein deficient diet may modify the turnover rate of membrane phospholipids and magnitude of phosphorylation of endogenous substrate proteins of PKC. The experiments were conducted on rats fed on three different types of laboratory prepared diets viz. casein (20% casein), deficient (4% protein, rice flour as source of protein) and supplemented (deficient diet supplemented with L-lysine and DL-threonine) for 28 days. The metabolism of phosphoinositides (PIs) and phosphatidyl choline (PC) was studied by equilibrium labeling with [<sup>3</sup>H] *myo* inositol and [<sup>14</sup>C methyl] choline chloride respectively. The phosphorylation of endogenous substrate proteins of PKC was studied by using <sup>32</sup>P- $\gamma$ -ATP followed by SDS-PAGE and autoradiography. The results suggest that in deficient group, there is an increased incorporation of [<sup>3</sup>H] *myo* inositol in PIs and inositol phosphate pool in comparison to the casein group. The phosphatidyl inositol (PI) turnover reduced, although there was a marginal increase in the phosphatidyl inositol monophosphate (PIP) and phosphatidyl inositol bis phosphate (PIP<sub>2</sub>). Supplementation of diet showed a reversal of the pattern towards control to a considerable extent. In the deficient group, PC metabolism showed an increased incorporation of [<sup>14</sup>C methyl] choline in choline phospholipids but decreased incorporation in phosphoryl choline in comparison with the casein group. The increase in total PC contents was significant but marginal in residue contents. The turnover rate of PC increased only marginally and that of residue declined. Supplementation of diet reduced the total contents of PC and residue, but the turnover rate of PC and residue remained still higher. Phosphorylation of endogenous proteins showed four different proteins of 78, 46, 33 and 16 kDa to be the substrates of PKC in casein group. In deficient group, phosphorylation of these proteins increased markedly while supplementation of diet had a reversing effect rendering the values to be intermediate between casein and the supplemented group. The changes in phospholipid metabolism and in phosphorylation of endogenous substrate proteins of PKC suggest that dietary protein deficiency causes alterations in transmembrane signalling mechanism in rat brain. These effects are partially reversed by improving the quality of proteins in the diet.

Feeding of protein deficient diet is known to impair the protein load and activity of several enzymes in brain and other tissues<sup>1,2</sup>. Activity of protein kinase C

(PKC) is reduced in brain of rats fed on diet deficient in quantity and quality of proteins which suggest changes in the transmembrane signalling in the tissue<sup>3</sup>. PKC is one of the key regulatory enzymes in transmembrane signalling pathways and its activation depends upon the generation of second messengers by hydrolysis of membrane phospholipids.

Initiation of transmembrane signalling is a function of receptor-ligand (stimulus) coupling. Receptor occupancy by stimulus leads to activation of membrane phospholipase C (PLC) which hydrolyzes membrane phosphatidyl inositol 4,5 bis phosphate (PIP<sub>2</sub>) to generate second messengers viz. sn-1,2 diacyl glycerol (DAG) and inositol 1,4,5 triphosphate (IP<sub>3</sub>). DAG is the physiological activator of PKC and IP<sub>3</sub> mobilizes calcium from endoplasmic reticulum

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which serves as an important cofactor for PKC activity. DAG can also be generated by hydrolysis of phosphatidyl choline (PC). PKC is predominantly cytosolic where it remains in the inactive form. The activation of PKC requires its translocation to the membrane in presence of raised levels of intracellular calcium where it binds to DAG and phosphatidyl serine (PS) to become fully active. Once activated, it phosphorylates substrate proteins which bring about the ultimate cell response<sup>4,6</sup>.

It is evident that membrane phospholipid turnover regulates the PKC activity. Hence changes in PKC activity after ingestion of diet deficient in quantity and quality of protein in diet as reported in our earlier communication<sup>3</sup> may be due to changes in turnover of membrane phospholipids viz. phosphoinositides and phosphatidyl choline. Further, alterations in PKC activity may influence the magnitude of phosphorylation of its intracellular substrate proteins. Therefore, experiments were conducted to delineate the changes in the phospholipid turnover and in phosphorylation of intracellular substrate proteins of PKC during the condition of dietary protein deficiency as reported in this communication.

### Materials and Methods

**Chemicals**—*myo* [2-<sup>3</sup>H] inositol (sp activity 18.9 Ci/mM) was purchased from Amersham International, Inc. UK. <sup>32</sup>P- $\gamma$ -ATP (sp activity 2500 Ci/mM) and [<sup>14</sup>C-methyl]-choline chloride (sp activity 45.5 mCi/mM) were purchased from BRIT, Bombay, India. L- $\alpha$ -phosphatidyl choline (PC), L- $\alpha$ -phosphatidyl ethanolamine (PE), L- $\alpha$ -phosphatidyl inositol (PI), phosphatidyl inositol 4-monophosphate (PIP), phosphatidyl inositol 4,5-bisphosphate (PIP<sub>2</sub>), L- $\alpha$ -lysophosphatidyl choline (LPC), histone III<sub>s</sub>, dithiothreitol (DTT), ethylene glycol-bis( $\beta$ -amino ethylene) tetra acetic acid (EGTA), phenylmethylsulphonyl fluoride (PMSF) etc. were purchased from Sigma Chemical Co. St Louis, USA. All other chemicals used were of analytical grade.

**Animals**—Male rats (40-50 g) of Wistar strain from Institute maintained animal colony were used.

**Diet**—Three different types of diet viz. casein, deficient and supplemented were prepared as described earlier<sup>3</sup>. Casein diet contained 20% protein (casein as source of protein) and served as standard diet. Deficient diet contained approximately 4% protein (rice flour as protein source). This diet was deficient both in quantity and quality of protein

because rice is deficient in two essential amino acids viz. L-lysine and DL-threonine. Supplemented diet was prepared to improve the quality by adding L-lysine (425 mg) and DL-threonine (360 mg) per 100 g to the deficient diet. All the diets prepared were isocaloric.

**Study design**—Animals were divided in three groups and designated as casein (C), deficient (D) and supplemented group (S). They were individually caged, paired under general condition of husbandry and mineral water *ad libitum*. Records of diet intake, body weight and dietary efficiency were maintained throughout the study as reported earlier<sup>3</sup>. After 28 days of feeding schedule, the animals were fasted for 12 hr before sacrifice.

### Studies on phospholipid metabolism:

**Phosphoinositide (PI) metabolism**—For PI turnover studies, after the feeding schedule of 28 days, each experimental rat was injected i.p. with *myo* [2-<sup>3</sup>H] inositol at the rate of 10  $\mu$ Ci/100 g body weight. The animals were sacrificed after 3 hr, brain removed surgically and homogenized in 4 volumes of chilled physiological saline. For phosphoinositide extraction, an aliquot of 0.5 ml homogenate was mixed thoroughly with 1.8 ml of acidified chloroform : methanol mixture (100 : 200 : 2 HCl, v/v/v). This was followed by addition of 0.6 ml of chloroform and 2M KCl solution each. The contents were thoroughly mixed on a vortex mixer and centrifuged at 500 g for 5 min at room temperature to separate the layers. Aqueous and organic phases were carefully separated in separate tubes. The precipitate was washed with 2 volumes of acidified chloroform : methanol and phases pooled with their respective phases. An aliquot of each of the organic and aqueous phase was processed for radioactivity counting using appropriate scintillation fluid for determination of total incorporation of *myo* [2-<sup>3</sup>H] inositol. Organic phase containing phospholipids was dried under the stream of N<sub>2</sub> and stored at 4°C till analyzed.

Phosphoinositides were resolved by thin layer chromatography (TLC) on silica gel G (thickness 400  $\mu$ ) impregnated with 1% oxalate and 1 mM EDTA. The TLC plates were activated at 110°C for 60 min followed by prerun in the solvent system consisting of chloroform: methanol: 4 N NH<sub>4</sub>OH (45 : 35 : 20; v/v/v) as described by Billah and Lapetina<sup>7</sup>. The samples (dried organic phases) were reconstituted in 100-200  $\mu$ l chilled chloroform and

loaded as streak in duplicates. Authentic standards (PIP<sub>2</sub>, PIP and PI) were co-chromatographed. After the run was complete the plates were removed, air-dried and exposed to iodine vapours. The corresponding spots were scraped. One band of each phosphoinositide was processed for scintillation counting and the other (duplicate) for total phosphorus determination by direct gel digestion method<sup>8</sup>. The data were expressed as picomoles of [<sup>3</sup>H] *myo*-inositol incorporate per g tissue and the phosphoinositide contents were expressed as µg phosphorus per g tissue.

**Phosphatidyl choline (PC) metabolism**—For studies on PC metabolism, one hour before sacrifice, rats of different dietary groups were injected i.p. with [<sup>14</sup>C-methyl]-choline chloride at the rate of 10 µCi/100 g body weight. Animals were sacrificed by cervical dislocation, brain removed surgically, washed in chilled physiological saline, extraneous adhering material removed, soaked on a filter paper, weighed and homogenized in 4 volumes of chilled physiological saline in a Polytron homogenizer. PC was extracted by the method described by Kirk *et al.*<sup>9</sup>. Accordingly, 0.5 ml homogenate was mixed with equal volume of 10% TCA containing 1 mM EDTA and centrifuged at 500 g for 5 min at room temperature. The pellet was washed with 1 ml of 5% TCA containing 1 mM EDTA, then with the same volume of distilled water and centrifuged. All the supernatants till this stage were discarded. The pellet was then extracted with 1 ml of chloroform : methanol : HCl (100 : 100 : 1; v/v/v) twice and both the extracts pooled. The pellet was further extracted with 0.6 ml chloroform: methanol: HCl (200 : 100 : 1; v/v/v). All these extracts were pooled to which 2.66 ml chloroform was added followed by addition of 1.95 ml of 0.1 M HCl. The contents of the tubes were thoroughly mixed on a vortex and centrifuged at 3000 g for 5 min at room temperature. The organic phase was collected, dried under the stream of N<sub>2</sub> and stored at 4°C till analyzed.

PC was resolved by thin layer chromatography on silica gel G (thickness 400 µ) as described by Abramson and Blecher<sup>10</sup>. The samples (dried organic phases) were reconstituted in 100-200 µl of chilled chloroform, applied in duplicate in a streak on a preactivated and prerun plate and transferred to the chamber saturated with chloroform: methanol : 7 N NH<sub>4</sub>OH (230 : 90 : 15; v/v/v). Standard PC was co-chromatographed. After the run was complete, the

plates were air dried, developed by exposure to iodine vapours and spots corresponding to PC scraped. Radioactivity counting and phospholipid phosphorus estimation was done similarly as for phosphoinositides mentioned above. The data were expressed as picomoles of [<sup>14</sup>C-methyl] choline incorporated per g tissue and the phospholipid contents expressed as µg inorganic phosphorus per g tissue.

**Protein phosphorylation**—Phosphorylation of endogenous proteins was carried out as described earlier<sup>11</sup>. The reaction mixture (40 µl) consisted of 50 mM tris HCl pH 7.5, 10 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 1 mM DTT, 50 µM ATP and <sup>32</sup>P-γ-ATP (1.0 µCi). In addition, 500 µM CaCl<sub>2</sub>·2H<sub>2</sub>O, 5 µg PS and 2 mM EGTA were added wherever required. Each set had an equal amount of protein (~80 µg). The reaction mixture was incubated at 30°C for 10 min. Reaction was stopped by addition of 10 µl of 4x treatment buffer (250 mM tris HCl, pH 6.8, 8% SDS, 40% glycerol and 20% β-mercaptoethanol). The samples were transferred to a boiling water bath for 2 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gel<sup>12</sup>. Standard marker proteins were simultaneously electrophoresed. The gels were stained with Coomassie blue R-250, dried on a gel drier and autoradiographed using X-ray films (Hindustan Photofilms Ltd., Bombay) with intensifying screens, at -70°C for 24 hr. Protein bands, phosphorylated by PKC, were identified by comparing the pattern in presence and absence of calcium and PS.

**Data analysis**—Student's *t* test for paired samples was applied for statistical analysis. *P* < 0.05 was considered significant.

## Results

**Metabolism of phosphoinositides**—The studies were designed to determine the incorporation of [<sup>3</sup>H] *myo*-inositol in phosphoinositides and inositols, total phospholipids phosphorus and turnover rate of phosphoinositides by equilibrium labelling experiments (the details are given in material and methods section). The radioactivity counting in organic and aqueous phases indicates the incorporation of *myo*-inositol in total phosphoinositides and inositol phosphates respectively. In casein group the total incorporation of [<sup>3</sup>H] *myo*-inositol in organic phase of brain was 0.36 ± 0.09 pico moles/g tissue. In deficient group, the incorporation of [<sup>3</sup>H] *myo*-inositol in total

phosphoinositides was observed to be raised significantly ( $P < 0.01$ ). Supplementation of deficient diet with L-lysine and DL-threonine amino acids was effective in reversing the pattern ( $P < 0.001$ ) (Table 1). Total incorporation of [ $^3\text{H}$ ] *myo*-inositol in aqueous phase representing incorporation in inositol phosphates in casein group was observed to be  $1.88 \pm 0.22$  pico moles/g tissue. In deficient group, the incorporation raised ( $P < 0.01$ ) in comparison with the casein group. In supplemented group, the values lie between casein and deficient group which indicate a reversal by the supplementation of deficient diet (Table 1).

The phosphoinositides were resolved by TLC into various components viz. phosphatidyl inositol bisphosphate ( $\text{PIP}_2$ ) phosphatidyl inositol monophosphate (PIP) and phosphatidyl inositol (PI). The total incorporation of [ $^3\text{H}$ ] *myo*-inositol, phospholipid phosphorus and turnover of each phosphoinositide were separately determined. In the casein group, the resolution pattern of phosphoinositide indicated the

concentration of  $\text{PIP}_2$  to be  $23.42 \pm 1.20$ , PIP to be  $23.34 \pm 3.2$  and PI to be  $195.10 \pm 57.17$   $\mu\text{g Pi/g}$  tissue constituting a total pool of about  $241.86$   $\mu\text{g/g}$  tissue (Table 2). The highest concentration and incorporation of [ $^3\text{H}$ ] *myo*-inositol was observed in PI followed by PIP and  $\text{PIP}_2$ . However, the turnover rate was maximum of PIP, which was comparable to  $\text{PIP}_2$ . In deficient group, the total pool of phosphoinositide increased to the tune of  $312.31$   $\mu\text{g/g}$  tissue. The PI concentration raised while the incorporation of *myo*-inositol declined and as such there was a reduction in turnover of PI in deficient group as compared to the casein group. A slight increase in incorporation and turnover in  $\text{PIP}_2$  and a decline in incorporation and turnover in PIP was also recorded in deficient group. In supplemented group, the total phosphoinositide pool showed recovery, returning the value to  $206.05$   $\mu\text{g/g}$  tissue. There was a remarkable reduction in total PI and some decline in incorporation due to which, there was a significant increase ( $P < 0.05$ ) in turnover rate in this group. Changes in  $\text{PIP}_2$  indicated

Table 1—Incorporation of [ $^3\text{H}$ ] *myo* inositol (picomoles per g tissue) in rat brain

[Values are mean  $\pm$  SD of 3 different observations]

	Casein	Deficient	Supplemented
Organic phase	$0.36 \pm 0.09$	$0.62 \pm 0.17^{**}$	$0.28 \pm 0.09^*$
Aqueous phase	$1.88 \pm 0.22$	$3.17 \pm 0.15^{**}$	$2.69 \pm 0.28$

$P$ : \*  $< 0.05$ , \*\*  $< 0.01$  as compared with the casein group.

Table 2—Phosphoinositide turnover in brain

[Values are mean  $\pm$  SD of 3 different observations]

		Casein	Deficient	Supplemented
$\text{PIP}_2$	a	$23.42 \pm 1.20$	$22.34 \pm 0.83$	$20.31 \pm 8.04$
	b	$97.94 \pm 13.48$	$99.60 \pm 10.8$	$80.43 \pm 2.52$
	c	$4.09 \pm 0.18$	$4.45 \pm 0.31$	$4.02 \pm 1.83$
PIP	a	$23.34 \pm 3.29$	$26.55 \pm 5.04$	$87.58 \pm 38.39$
	b	$109.63 \pm 10.59$	$96.12 \pm 12.17$	$106.85 \pm 17.14$
	c	$4.72 \pm 0.45$	$3.66 \pm 0.58$	$1.33 \pm 0.38^{**}$
PI	a	$195.10 \pm 57.17$	$263.42 \pm 61.09$	$98.16 \pm 11.98$
	b	$139.82 \pm 30.73$	$130.48 \pm 10.95$	$113.53 \pm 18.83$
	c	$0.72 \pm 0.05$	$0.50 \pm 0.07$	$1.16 \pm 0.23^*$

$\text{PIP}_2$  : phosphatidyl inositol 4,5 bisphosphate; PIP : phosphatidyl inositol 4 - monophosphate; PI : phosphatidyl inositol.

a:  $\mu\text{g}$  phosphoinositide phosphorus per g tissue; b: femto moles [ $^3\text{H}$ ] *myo*-inositol incorporated per g tissue; c: turnover of phospholipid (femto moles of [ $^3\text{H}$ ] *myo*-inositol incorporated per  $\mu\text{g}$  phospholipid phosphorus)

$P$ : \*  $< 0.05$ , \*\*  $< 0.01$  as compared with the casein group.



a reversal of change caused due to deficiency in quantity and quality of protein in the diet. The total PIP levels were observed to be remarkably high and therefore the turnover rate declined significantly ( $P < 0.01$ ) (Table 2).

**Metabolism of phosphatidyl choline (PC)**—Studies were designed similarly as for phosphoinositides. [ $^{14}\text{C}$ -methyl]-choline chloride was used for equilibrium labelling to trace the turnover of PC in brain. The organic and aqueous phases of each tissue were separated and radioactivity determined to evaluate the total incorporation in choline phospholipids and phosphoryl choline respectively. The phospholipid fraction was then subjected to thin layer chromatography on silica gel G to resolve the PC for total contents and incorporation of [ $^{14}\text{C}$ -methyl]-choline chloride in it. Incorporation of [ $^{14}\text{C}$ -methyl]-choline chloride in organic phase in casein group in brain was observed to be  $96.65 \pm 18.28$  pico moles per g tissue. In deficient group, there was an increase in [ $^{14}\text{C}$ -methyl]-choline chloride incorporation in comparison with casein group. Supple-

mentation of diet with L-lysine and DL-threonine, however, further raised it to a highly significant ( $P < 0.001$ ) level (Table 3). In aqueous phase, in casein group, the incorporation of [ $^{14}\text{C}$ -methyl]-choline chloride was observed to be  $253.76 \pm 62.38$  pico moles per g tissue. In deficient group, the incorporation decreased in comparison with casein group. Supplementation of deficient diet with lysine and threonine led to reversal of pattern (Table 3).

The phosphatidyl choline turnover revealed that in the casein group, the total PC was  $397.57 \pm 99.00$  and the residue was  $585.57 \pm 29.82$   $\mu\text{g Pi/g}$  tissue. In deficient group, the total PC remarkably increased with a significant ( $P < 0.01$ ) increase in incorporation of [ $^{14}\text{C}$ -methyl]-choline chloride. There was a marginal increase in the turnover rate of PC also in this group in comparison with the casein group. The total Pi contents of residue were still higher than the PC contents, which retained considerably high radioactivity and turnover rate in casein group. In deficient group, total Pi of residue increased marginally, and incorporation declined leading to a

Table 3—Incorporation of [ $^{14}\text{C}$  methyl] choline chloride in rat brain

[Values expressed as picomoles per g tissue are mean  $\pm$  SD. Figures in parentheses are no. of observations]

	Casein	Deficient	Supplemented
Organic phase	$0.36 \pm 0.09$ (4)	$0.62 \pm 0.17^{**}$ (3)	$0.28 \pm 0.09^*$ (3)
Aqueous phase	$1.88 \pm 0.22$ (3)	$3.17 \pm 0.15^{**}$ (4)	$2.69 \pm 0.28$ (3)

$P$ : \*  $< 0.05$ , \*\*  $< 0.001$  as compared with the casein group

Table 4—Phosphatidyl choline (PC) turnover in brain

[Values are mean  $\pm$  SD. Figures in parentheses indicate no. of observations]

		Casein	Deficient	Supplemented
PC	a	$397.57 \pm 99.00$ (3)	$669.90 \pm 192.98$ (4)	$414.49 \pm 97.33$ (4)
	b	$26.31 \pm 5.11$ (3)	$52.94 \pm 4.27^{**}$ (4)	$142.05 \pm 29.08^{**}$ (4)
	c	$0.06 \pm 0.004$ (3)	$0.08 \pm 0.02$ (4)	$0.35 \pm 0.108$ (4)
Residue <sup>1</sup>	a	$585.57 \pm 29.8$ (4)	$669.15 \pm 170.04$ (4)	$541.77 \pm 65.12$ (4)
	b	$111.22 \pm 16.45$ (3)	$85.89 \pm 9.97$ (4)	$331.39 \pm 93.60^*$ (4)
	c	$0.18 \pm 0.01$ (4)	$0.13 \pm 0.02^*$ (4)	$0.51 \pm 0.07^{**}$ (3)

<sup>1</sup>Residue-material left at the starting point of TLC lane.

a: phospholipid phosphorus ( $\mu\text{g}$  per g tissue); b: incorporation of [ $^{14}\text{C}$ -methyl] choline chloride (pico moles per g tissue); c: pmole of [ $^{14}\text{C}$ -methyl] choline chloride incorporated per  $\mu\text{g}$  phospholipid phosphorus.

$P$ : \*  $< 0.05$ , \*\*  $< 0.01$  as compared with the casein group

significant reduction ( $P < 0.05$ ) in its turnover in comparison with the casein group. Supplementation of diet although helped in reducing the total PC contents of brain towards the casein group, but a highly significant increase ( $P < 0.01$ ) in incorporation of [ $^{14}\text{C}$ -methyl]-choline chloride was observed which led to significant ( $P < 0.05$ ) increase in its turnover rate as well. In residue, a similar effect was observed in total Pi contents but contrary to deficient group, there was a significant ( $P < 0.05$ ) increase in incorporation of choline chloride leading to a significant increase ( $P < 0.01$ ) in its turnover rate (Table 4).

**Protein phosphorylation**—Protein phosphorylation was carried out using  $^{32}\text{P}$ - $\gamma$ -ATP. The proteins were resolved on SDS-PAGE, gels dried and autoradiographed. For identifying the proteins phosphorylated by PKC, the pattern of phosphorylation of bands in presence and absence of  $\text{Ca}^{2+}$ /PS was compared. The lanes were then individually scanned in a Shimadzu scanner CS 9000 and quantitated on the basis of optical density of the band of interest.  $\Delta$  O.D. was calculated by subtracting

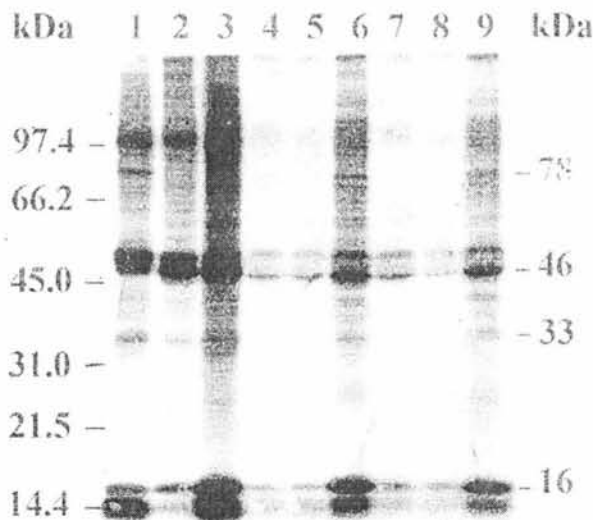


Fig. 1—Autoradiogram showing protein phosphorylation by protein kinase C in rat brain. [Left panel represents molecular weights of standard marker proteins. Right panel represents molecular weights of substrate proteins of protein kinase C. Details of lanes. Casein group—Lane 1 to 3 : Lane 1—in absence of calcium and phosphatidyl serine, Lane 2—in presence of calcium, Lane 3—in presence of calcium and phosphatidyl serine. Deficient group—Lane 4 to 6 : Lane 4—in absence of calcium and phosphatidyl serine, Lane 5—in presence of calcium, Lane 6—in presence of calcium and phosphatidyl serine. Supplemented group—Lane 7 to 9 : Lane 7—in absence of calcium and phosphatidyl serine, Lane 8—in presence of calcium, Lane 9—in presence of calcium and phosphatidyl serine.]

the O.D. of bands in control lane (in absence of  $\text{Ca}^{2+}$  and PS) from the O.D. of bands phosphorylated in presence of  $\text{Ca}^{2+}$  and PS. The autoradiogram showed that PKC phosphorylated four different proteins of 78, 46, 33 and 16 kDa in brain in all the groups (Fig. 1). Scanning of the bands of individual lane in various groups showed that these proteins correspond to peaks marked as 1, 2, 3 and 4 respectively (Fig. 2). The  $\Delta$  O.D. of each of these peaks is given in Table 5. In deficient group,  $\Delta$  O.D. of all the peaks showed a marked increase indicating increased phosphorylation by PKC in comparison with casein group. Supplementation of diet with L-lysine and DL-threonine led to reversal of the affect, rendering all the  $\Delta$  O.D.s to be intermediate between casein and deficient group.

## Discussion

Balanced diet is essentially required for normal growth and development of the body. If the protein portion of diet is poor in quality and quantity then

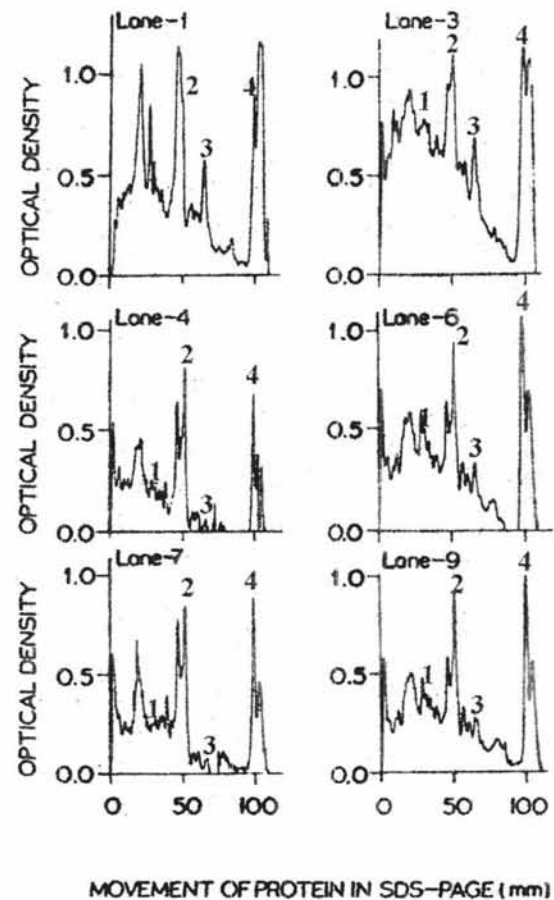


Fig. 2—Scanning pattern of lanes of autoradiogram at 560 nm. [Details of lanes are same as in Fig. 1. Peaks 1 to 4 represent proteins phosphorylated by protein kinase C.]

Table 5—Phosphorylation of proteins by PKC in brain

Peak No.	Mol Wt. (kDa)	$\Delta$ O.D.*		
		Casein	Deficient	Supplemented
1	78	0.60	0.95	0.48
2	46	0.44	0.91	0.67
3	33	0.19	0.61	0.45
4	16	0.44	1.42	0.90

\* Difference in optical density ( $\lambda$  560 nm) of protein bands phosphorylated in presence and absence of  $\text{Ca}^{2+}$  and phosphatidyl serine.

there are reduction in food intake, feed efficiency and growth<sup>13-15</sup>. Feeding of diet poor in quantity and quality of protein caused a remarkable reduction in the weight gain of animals<sup>3</sup>. The total protein contents of brain declined marginally (Bansal *et al.*, unpublished data) which may be attributed to the changes in protein synthesizing system and polyribosome profile of endoplasmic reticulum<sup>16,17</sup>. A decrease in protein synthesis and ribosome aggregation has been reported by Yokogoshi *et al.*<sup>18</sup> in rat brain when the ingested diet was poor in quality and quantity of protein. The quality of the protein can be improved by supplementation of the deficient diet appropriately with the addition of the limiting amino acids. Beverly *et al.*<sup>19</sup> reported that supplementation of the diet with limiting amino acids leads to increased protein synthesis in prepyriform cortex of rats fed on imbalance diet. An altered protein synthesis in brain may lead to alteration in the enzyme make up thereby influencing cellular metabolism and several pathways. These changes may ultimately lead to varied cell response to various stress conditions caused by stimuli. The magnitude of the response depends upon the signalling system operating via second messengers<sup>6</sup>. One of the major signalling pathways involves hydrolysis of membrane phosphatidyl inositol 4,5-bisphosphate ( $\text{PIP}_2$ ) thereby generating inositol 1, 4, 5-triphosphate ( $\text{IP}_3$ ) and sn 1, 2 diacylglycerol (DAG)<sup>4</sup>.  $\text{IP}_3$  and DAG bring about activation of protein kinase C (PKC)<sup>20,21</sup>, which in turn phosphorylates protein(s), leading eventually to the cell response<sup>4</sup>.

The generation of  $\text{IP}_3$  and DAG formed by the hydrolysis of membrane phosphatidyl inositol<sup>22,23</sup> leads to activation of PKC which is the key regulatory enzyme in this pathway<sup>4</sup>. In the present study, the phosphoinositide turnover revealed an increase in incorporation of [ $^3\text{H}$ ] *myo*-inositol in the phosphoinositide pool in the rat brain in deficient group which is contrary to the findings of Anantha *et al.*<sup>24</sup>. The

increased incorporation of [ $^3\text{H}$ ] *myo*-inositol was associated with increased turnover of  $\text{PIP}_2$  and decreased turnover of PIP and PI, though there was minor decrease in total  $\text{PIP}_2$  mass. This suggests that dietary protein deficiency led to increased synthesis of  $\text{PIP}_2$  and also that its rate of degradation was higher than its synthesis. In case of PIP and PI, it was vice-versa of  $\text{PIP}_2$  in brain, indicating activation of other kinases towards PIP and PI metabolism. This pattern indicates an increase in PI-PLC activity and gets support from increased incorporation of *myo*-inositol in aqueous phase, which represents inositol phosphate pool. Since  $\text{IP}_3$  of inositol phosphate mobilizes calcium from intracellular stores<sup>6</sup>, it may explain the translocation of PKC from cytosol to membrane. These changes in the phosphoinositide pool may be responsible for change in PKC activity in brain in protein deficient group reported by us earlier<sup>3</sup>.

DAG is generated by hydrolysis of  $\text{PIP}_2$  and PC<sup>5</sup>. In the present study it is apparent that  $\text{PIP}_2$  hydrolysis in brain in deficient group may contribute to the formation of DAG. For long term affect, DAG may be formed by hydrolysis of PC directly by the action PC-PLC<sup>25,26</sup> or PC may be first hydrolysed by phospholipase D in brain, resulting in formation of phosphatidic acid, which is then converted into DAG by the action of phosphatidic acid monoesterase<sup>5</sup>. In the present study, PC turnover indicates an increase in synthesis of PC in deficient group. There was no substantial change in turnover rate, indicating simultaneous degradation of PC. Since anabolic and catabolic rates are balanced and anabolism of PC is increased, it indicates a net increase in catabolic rate of PC, suggesting increased generation of DAG. It is thus evident that in protein deficiency condition, phospholipid metabolism plays an important role in PKC status and its activation in brain.

Once activated, PKC may phosphorylate a number of tissue proteins for the cellular functions to occur<sup>4,5</sup>.

Rat brain has been reported to consist of a mixture of proteins and glycoproteins ranging from 200 to 15 kDa present in functionally distinct segments viz. axolemma (the perikaryal plasma membrane), dendritic and synaptic membranes<sup>27</sup>. In the present investigation, four different proteins of 78, 46, 33 and 16 kDa were identified to be phosphorylated by PKC in brain which may represent to be some of these proteins. The phosphorylation of all these proteins was increased in the protein malnutrition, which tended to reverse when the diet was supplemented with L-lysine and DL-threonine. The changes in the phosphorylation of these proteins in dietary protein deficiency may alter the functions of the brain associated with these proteins thereby influencing its response under conditions of stress. The quality of proteins depends upon its amino acid composition. In the present study rice flour was used as the source of protein in the deficient diet which is limiting in L-lysine and DL-threonine amino acids<sup>28,29</sup>. The quality of the diet was improved by appropriately adding these amino acids into the deficient diet to constitute the supplemented diet. Feeding of such diet had a considerable effect on reversing the alterations in the parameters studied for evaluation of mechanism of signal transduction in brain. This may be due to the amino acid supply, which can influence neuronal proteins synthesis for which direct evidence has been given by Parks *et al*<sup>30</sup>. This also gets support from the studies conducted by Beverly *et al*<sup>19</sup> that supplementation of diet with the limiting amino acid apparently increases the protein synthesis in the prepyriform cortex of rats. Thus improvement in quality of protein can considerably help in improving the impairment of brain response to the stimulus caused due to the poor quality of dietary proteins.

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