C H A P T E R III

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

.....

1. Effects of Prenatal Undernutrition at Birth :

As mentioned earlier, for the studies on prenatal undernutrition female rats were fed low protein (5%) diet from the first day of conception as determined by presence of sperms in the vaginal lavages. The control females were similarly fed 20% protein diet ad libitum. Adverse effects were seen on the reproductive performance of the protein deficient animals (Table 14). There was a greater incidence of still births and higher mortality during postnatal period. Although, the average litter size in the control group was almost similar, deaths during neonatal period were almost ten times more in the low protein group as compared to that with the control. Although average birth weight of the litter in the protein deficient group was slightly less than that in the control, the average weaning weight in the deficient animals was almost four times less than that in the control. However, a sufficient number of viable pups survived till the weaning age.

Maternal protein deficiency during gestation has been reported to be associated with smaller fetuses with reduced bone development and increased number of still births, however, gestational age and litter size were unaffected (Nelson and Evans, 1953; Venkatachalam and Ramnathan, 1964;

Table- 14 : Effects of a low protein diet on reproductive performance*

	% Protein in the Diet	
	20	5
1. No. of females kept for breeding	22	38
2. No. of pregnancies	19	32
3. % Fertility	85	84
4. Average litter size	8	7.5
5. No. of pups still born	2	14
6. No. of live births	152	236
7. No. of deaths during neonatal period	8	88
8. % Mortality	5	37
9. Average birth weight (g)	6 ± ?	4.5 ± ?
10. Average weaning weight (g)	45	12

* Dietary regimen was started from first day of pregnancy.

Table-15 : Effects of maternal protein deficiency during gestation on prenatal development at birth[1].

	% Protein in maternal diet		$\frac{LP}{HP}$ X 100
	20 (HP)	5 (LP)	
No. of pups[2]	13	26	
Body weight (g)	6.1±0.14	4.4±0.05*	72*
Brain weight (g)	0.269±0.01	0.208±0.01*	77*
$\frac{\text{Brain weight}}{\text{Body weight}}$	0.044±0.001	0.047±0.001**	107**
Values per g Brain :			
Protein (mg)	56.8±0.9	55.9±1.1	99
DNA (mg)	1.90±0.05	1.91±0.04	101
$\frac{\text{Protein}}{\text{DNA}}$	29.4 ±0.4	29.3±0.5	99
AChE[3]	2.20 ±0.07	1.88±0.05‡	85‡
ChAc[4]	0.36 ±0.02	0.32±0.02	89

Values are expressed as Mean ± S.E.M.

1. Dams were fed respective diets from first day of conception.
2. 3 to 4 brains were pooled for estimation.
3. µmoles of acetylthiocholine iodide hydrolyzed/min/g of brain.
4. µmoles of ^{14}C ACh formed per hr. per g brain.

Values marked with astericks significantly differ from control values.

* $p < .001$; ** $p < .05$; ‡ $p < .01$.

that the growth of the brain, perhaps, does not parallel that of whole body weight.

The protein concentration of the brain was not found to be altered, a finding consistent with that of several other investigators (Zamenhof et al., 1968; Zeman and Stanbrough, 1969; Balázs and Patel, 1973; Envonwu and Glover, 1973; Telang, 1975). The concentration of DNA was also not found to be affected in the progeny of the deficient mothers. However, deficits in total DNA content have been reported by Zamenhof et al. (1971).

The activity of brain acetylcholinesterase (AChE) was significantly affected with a deficit of 15%. However, choline acetylase (ChAc) activity was not significantly reduced. Adlard and Dobbing (1971a; 1971b) reported no change in the activities of enzymes such as succinic dehydrogenase, aldolase, AChE and β-N-acetyl glucosaminidase in pups born of undernourished mothers. However, in their studies dams were underfed by 50% from 7th day of gestation onwards. The new born pups had a body and brain weight deficits of 12 and 8 % respectively as against 28 and 23 % observed in the present studies. Thus, the reduced activity of AChE observed in the progeny of the deficient mothers could be, perhaps, attributed to much more drastic reduction in the body and brain weights at birth. This deficit would

suggest a possible reduction in number of synapses or alteration in the synaptic structure. It is interesting to note that the appearance of reflex activities such as righting, negative geotaxis, cliff avoidance and other development parameters reported to be delayed in the retarded pups at birth (Simonson et al., 1968). In conclusion, it can be said that prenatal undernutrition induced by feeding the mothers a low protein diet during gestation resulted in significant deficits in body and brain weights and activity of AChE in the brain of the progeny at birth.

2. Effects of Neonatal Undernutrition at Different Ages :

As mentioned earlier, neonatal undernutrition was induced either by manipulating litter size soon after partus or by feeding low protein (5%) diet to mothers from the first day post partus.

Pups reared in standard or large litter size of 8 or 16, were killed at 7, 14, 21 and 28 days after birth for estimation of brain ACh. Also some pups were killed at birth for base line data. The results are presented in Table 16. It can be seen that undernutrition during the neonatal period achieved by manipulation of litter size resulted in significant deficits in body and brain weights

Table- 16: Effects of neonatal undernutrition on brain ACh at different ages[1]

Age (in days)	Body wt.(g)		Brain wt. (g)		Acetylcholine[2]			
					µg/brain		µg/g brain	
	SL	LL	SL	LL	SL	LL	SL	LL
At birth	5.0± 0.04		0.214±0.014		0.14±0.04(4)		0.63±0.02	
7	11.0±0.4	9.0±0.2*	0.68 ±0.01	0.62 ±0.05	1.17 (2) ±0.15	1.00 (4) ±0.04	1.71 ±0.20	1.63 ±0.02
14	16.0±1.0	11.0±0.3*	0.96 ±0.03	0.84* ±0.02	1.32 (4) ±0.05	1.07 (6) ±0.05	1.39 ±0.06	1.24 ±0.05
21	28.0±1.4	18.0±1.2*	1.25 ±0.03	1.13* ±0.02	1.55 (6) ±0.14	1.35 (7) ±0.10	1.23 ±0.10	1.19 ±0.10
28	60.0±0.2	34.0±1.4*	1.41 ±0.07	1.22* ±0.07	2.57 (6) ±0.17	2.22 (7) ±0.18	1.80 ±0.10	1.80 ±0.14

Values are expressed as Mean ± S.E.M.

SL = Small litter size of eight; LL = Large litter size of sixteen.

1. This work was published in J. Neurochemistry, 23, 119-121 (1974). Rajalakshmi et al

2. As acetylcholine chloride. * ($p < 0.01$)

Number of estimations shown in parenthesis.

from 14 days onwards. The deficits in the body weights at 7, 14, 21 and 28 days of age were approximately 18, 30, 50 and 55 % as compared to controls. The respective deficits in brain weights were 9, 12, 10 and 14 %. In other words, although neonatal undernutrition was found to result in a progressive retardation in body weight, similar retardation in brain weight was not observed. Similar observations were reported by Swaiman et al.(1971), Telang (1975) and by other workers.

It is interesting to note that ACh concentration increased with age. There was 300 fold increase at 28 days. Eventhough the body weight and brain weight deficits were apparent from the 7-14 days of life, ACh concentrations were not affected throughout the length of study. This contrasts with the lower concentration of brain lipids, with a similar degree of neonatal undernutrition (Dobbing, 1968; Guthrie and Brown, 1968). However, the ACh content of the brain was slightly lower at all ages, the decrease was statistically significant only at 14 days and was in proportion to the decrease in brain weight. These results can possibly be interpreted to mean that the neonatal undernutrition so achieved failed to affect ACh concentration, particularly during the most vulnerable period of the brain development. Decrease in maternal stimulation because of increase in litter size could possibly counteract the

effects of undernutrition. It is interesting to note that Sereni et al. (1966) reported decreased concentrations of 5-OH, tryptamine and norepinephrine in similarly undernourished rats at 16 days of age but subsequently the concentrations returned to normal in spite of continued undernutrition.

Effects of neonatal undernutrition achieved by manipulation of litter size on the cholinergic enzymes were studied at 28 days. The results are summarised in Table- 17. The deficit in body and brain weights was similar to that of presented earlier. However, concentrations of protein and DNA were not found to be affected. Similar observations have been made by Swaiman et al. (1970); Envonwu and Glover, (1973) and Sobotka et al. (1974). Nevertheless the contents of whole brain would be definitely much lower in the large litter size as there is appreciable reduction in the brain weight. Similarly, the activities of AChE and ChAc were unaffected. Sereni et al. (1966) reported significantly reduced activity of AChE in similarly undernourished pups at 8 and 14 days; but at day 21 deficit was much less and statistically not significant. Im et al. (1971a) reported similar observations, with neonatally undernourished pups by feeding low protein diet (12 %) to mothers from the first day post-partus. However, as mentioned elsewhere, more

Table-17 : Effects of neonatal undernutrition on cholinergic enzymes of rat brain at 28 days[1].

	Litter size		$\frac{UN}{N}$ x 100
	(8) Small	(16) Large	
No. of pups	8	16	
Body weight (g)	60.000±1.1	34.000±1.2*	57
Brain weight (g)	1.415±0.07	1.231±0.021*	87
$\frac{\text{Brain wt.}}{\text{Body wt.}}$	0.024±.0003	0.037±0.001*	65
Values per g brain			
No. of observations	8	8	
Protein (mg)	87.000±1.9	88.200±1.1	99
DNA (mg)	2.140±0.15	2.200±0.04	94
$\frac{\text{Protein}}{\text{DNA}}$	41.700±1.7	43.700±0.7	105
AChE[2]	9.500±0.30	9.900±0.34	104
ChAc[3]	3.770±0.08	3.630±0.11	96

Values are expressed as Mean ± S.E.M.

1. Neonatal undernutrition (UN) was achieved by increasing litter size to 16 pups per dam. Both groups were fed 20% protein diet ad lib.
2. µmoles acetylthiocholine iodide hydrolyzed per min per g.brain.
3. µmoles of ^{14}C-ACh formed per hr. per g of brain, values marked with astericks significantly different from control values.

   * ($p < 0.001$).

severe degree of undernutrition instituted at different stages of brain maturation had different effects on the activities of AChE and ChAc. Moreover, activity of AChE has been known to be affected by early handling (maternal stimulation) of pups (Tapp & Markowitz 1965) associated with emotional reactivity (Denenberg, 1964). The effects of improvised and enriched environment on the enzyme activity in rat brain are very well documented (Rosenzweig et al., 1972). Increasing the amount of environmental stimulation is known to partially ameliorate the alteration in behaviour due to malnutrition. (Yatkin and McLaren, 1970; Levitsky and Barnes, 1972).

Eckhert et al. (1976a) reported lower activity of ChAc in both the brain stem and cerebellum of rats undernourished during both neonatal period (by maternal protein deficiency) and post-weaning period. Contrary to these findings, Gambetti et al. (1972) failed to find any change in activity of AChE at 12 and 24 days, in brain cortex of pups of mothers fed protein-deficient diet from 10th day of gestation. However, the activity was increased at 24 days in recovered synaptosomal fractions of brain cortex of the undernourished pups. Moreover, the increased activity of ChAc and protein levels in the recovered synaptosomal fraction were also observed by these workers.

In conclusion, the present results suggest that neonatal undernutrition achieved by manipulation of litter size do not seem to affect cholinergic system notwithstanding the fact that both body and brain weights were adversely affected. However, the possibility of specific changes taking place in individual components of the cholinergic system in different regions and subcellular fractions of the brain cannot be ruled out. The difference in maternal stimulation in this design of experiment is another factor which could offset the effects of undernutrition on the cholinergic system.

3. Effects of Pre-and/or Post-natal Undernutrition at different ages :

Since neonatal undernutrition achieved by manipulation of litter size was not found to affect the cholinergic system, it was felt worthwhile to study different mode of achieving pre-natal and/or post-natal undernutrition. For this purpose, the female rats were fed low protein diet (5 %) from the first day of conception till the end of weaning (G^-L^-). The controls were fed 20% protein diet ad libitum (G^+L^+). The third group was fed 20% protein diet during gestation and 5% protein diet from first day post-partus (G^+L^-). The idea was to achieve more severe degree of undernutrition at two different stages of brain

maturation. Secondly, the possibility of differences in maternal stimulation due to differences in the litter sizes offsetting the effects of undernutrition was aimed to be studied.

It is important to mention at this stage that feeding mothers a low protein diet during lactation results in diminished milk production but does not affect the composition of milk (Perisse and Salmon-Legagnear, 1960; Chow and Lee, 1964) resulting into reduction in absolute amounts of protein and calories (Mueller and Cox, 1946) available to pups. However, percent protein calories would remain essentially same. Maternal protein deficiency during gestation, as mentioned earlier, results into the reduction of body and brain weight of the progeny.

The results of these studies on brain ACh concentration at 21 days are presented in Table- 18. Whereas, body weights were affected almost to the same extent (i.e., 27 and 29 % of the normal in the G^-L^- and G^+L^- groups respectively) the brain weights were markedly lower in G^-L^- group (74% of normal) in comparison with that of G^+L^- group (84 % of normal). Thus, protein restriction during lactation superimposed on a stress of gestational protein deficiency seems to affect brain weight markedly. In this connection, it is interesting to recall that the similar deficits in

Table-18 : Effects of maternal protein deficiency during gestation and/or lactation on brain ACh levels of the progeny at 21 days of age.

	+ Dietary Regimen +		
	G^+L^+	G^+L^-	G^-L^-
No. of pups	6	6	6
Body weight (g)	45.000±1.3	13.000±1.0*	12.000±0.8*
Brain weight (g)	1.391±0.008	1.167±0.035*	1.033±0.032*
ACh (µg)			
Whole brain	2.200±0.10	1.610±0.13*	1.280±0.12*
g. Brain	1.600±0.05	1.370±0.08**	1.220±0.09***

Values are expressed as Mean ± S.E.M.

G^+L^+ = 20% protein diet during gestation and lactation

G^+L^- = 20% protein diet during gestation and 5% protein diet during lactation.

G^-L^- = 5% protein diet during both periods.

Values marked with astericks significantly differ from the controls.

* ($p < 0.001$)

** ($p < 0.05$)

*** ($p < 0.01$)

neonatally undernourished rats by increasing the litter size, were 36 and 10 % respectively. The more drastic reduction observed in body weights of the undernourished groups (G^+L^- and G^-L^-) over those of pups reared in large litters needs some elaboration. It is not surprising as the mother nursing a large litter can compensate to some extent by increasing food intake which is reduced with protein deficiency. The food intakes of mothers with standard and large litters were 32 g and 45 g as against 8 - 12 g in mothers fed a low protein diet. Similar deficits have been reported by several other workers besides Telang (1975) and Nakhasi (1975).

In other words, the effects of undernutrition were greater on brain weight with maternal protein deficiency than with increased litter size and somewhat greater when maternal protein deficiency was induced during both gestation and lactation than during only lactation. ACh content and concentration were found to be significantly affected in the severely undernourished pups. The deficits in the concentration were 14 and 24 % in G^+L^- and G^-L^- respectively. Similarly the respective deficits in ACh content work out to be 27 and 42 %. This is contrary to the earlier observations with mild neonatal-undernutrition achieved by increasing the litter size. However, as mentioned earlier, the difference in maternal stimulation

due to altered litter size is a factor to be considered which could offset the possible effect of undernutrition. In addition to this factor, the degree of severity and onset of undernutrition are other possible factors which could influence the maturation of cholinergic system and its components.

As mentioned earlier brain ACh concentration increases with age, the finding is in agreement with that of Naik et al. (1970). This would suggest maturation of cholinergic system taking place during this period. This parallels the period of multiplication of microneurons and synaptogeneses till 21 days of age in rats. The deficits in ACh concentration may be a reflection of a possible delayed synaptogenesis. However, there is a need to work out more carefully a correlation between ACh concentration with cholinergic enzymes (Rosenzweig et al., 1972) and synaptogenesis before arriving at any positive conclusion.

In this connection, it is to be noted that Sereni et al. (1966) found transitory reduction in the levels of 5-OH-tryptamine and norepinephrine at 16 days in undernourished rats. Subsequently, these deficits were normalised with continued undernutrition. This aspect of transitory reduction in a level of a neurotransmitter and subsequent

adaptation with age needs to be studied in details in connection with ACh level. It is also interesting to note that the concentrations of neurotransmitter amines were found to be significantly lowered at 14 days of age and onwards in rats born of mothers fed protein deficient diet during gestation and lactation (Ramanamurthy, 1977).

The finding of a lower brain ACh levels with severe undernutrition contrasts with the increase reported in starvation (Naik et al., 1970). This may be because the effects of chronic undernutrition are different from those of complete starvation. It is interesting to note that a low protein diet was found to produce effects different from those of a protein free diet (Rajalakshmi and Ramakrishnan, 1972). Besides what is more relevant is the vulnerable period of brain maturation during neonatal period and the stage at which severe nutritional insult is inflicted.

In conclusion, it can be said that the more severe degree of undernutrition during gestation and/or lactation reduced brain ACh ~~component~~ concentration as compared to neonatal undernutrition induced by increasing the litter size.

Since undernutrition during pre- and post-natal period was found to result in significant deficits in the

ACh concentrations, it was necessary to study the profiles of protein and DNA levels and the activities of cholinergic enzymes, AChE and ChAc, at 7, 14 and 21 days of age.

Undernutrition induced from the first day of gestation (G^-L^-) had more adverse effect on body and brain weights as compared to neonatal undernutrition (G^+L^-) (Table 19). The body weight deficits for G^+L^- group at 7 and 14 day were 41 and 59% respectively; the brain weight deficits were 11 and 26 %. The figures for G^-L^- group were 59 and 70 % for body weight deficit as against 30 and 42 % for brain deficits. Similarly, the values for the ratio of brain weight/body weight were significantly elevated for G^+L^- and G^-L^- at 7 and 14 days. The interesting fact to be noted is that deficits for G^-L^- were more as compared to G^+L^- group. The ratio of the two weights for G^+L^- at 7 and 14 days were 0.068 $\pm$ .003 and 0.082 $\pm$ .001 as against the values for G^-L^- were 0.071 $\pm$ .003 and 0.087 $\pm$ .001. This suggests, as pointed out earlier, that the brain growth does not parallel with that of body growth and in this respect G^-L^- group has a more of retardation as compared to G^+L^- group.

The concentrations of protein and DNA were not significantly reduced. The concentrations were found to

Table-19 : Effects of maternal protein deficiency during gestation and/or lactation on the body weight, brain weight, brain protein & DNA levels of the progeny at different ages.[1]

Age (days)	Dietary regimen	Body wt. g.	Brain wt. g.	Brain wt. / Body wt.	Protein mg/g.	DNA mg/g	Protein-DNA
7	G^+L^+ (8)[2]	17 ± 0.7	0.734±0.014	0.042±0.001	58.00±0.7	2.03±0.08	28.7± 0.7
	G^+L^- (8)	10 ± 0.6*	0.655±0.012*	0.068±0.003*	57.50±0.8	1.91±0.07	30.4± 1.0
	G^-L^- (8)	7 ± 0.5*	0.513±0.010*	0.071±0.003*	57.70±0.8	1.90±0.04	30.0± 0.5
14	G^+L^+ (7)	27.± 0.4	1.204±0.034	0.044±0.001	76.10±1.9	2.15±0.13	35.4± 2.0
	G^+L^- (8)	11 ± 0.8*	0.896±0.054	0.082±0.001*	74.80±2.0	2.07±0.05	36.2± 0.8
	G^-L^- (8)	8 ± 0.4*	0.704±0.02*	0.087±0.001*	73.67±1.9	2.04±0.05	36.1± 0.9
21	G^+L^+ (6)	44 ± 0.9	1.38 ±0.014	0.032±0.001	96.7 ±2.8	2.27±0.11	42.8± 1.9
	G^+L^- (7)	13 ± 0.7**	1.11 ±0.30**	0.086±0.003**	95.0 ±1.5	2.13±0.04	44.7± 1.2
	G^-L^- (7)	12 ± 0.5**	1.04 ±0.033**	0.086±0.004**	94.6 ±1.3	2.10±0.03	45.0± 0.9

Values are expressed as Mean ± S.E.M.

1. Dams were fed respective diets from the first day of conception or first day post-partus till 21 days after partus. G^+L^+ = 20% protein diet during gestation and lactation; G^+L^- = 5% protein diet during lactation; G^-L^- = 5% protein diet during gestation and lactation.

2. Numbers in parenthesis indicate number of observations.
Values marked with asterisk are significantly different from the control.
* ($p < 0.01$) ; ** ($p < 0.001$).

increase with the age. However, the ratio of protein to DNA was slightly higher in G^-L^- and G^+L^- group as compared to that of G^+L^+ group. But this difference was not statistically significant.

The deficits in body and brain weights were further increased at 21 day; the deficits being 70, 20 % and 73, 25 % respectively for G^+L^- and G^-L^-. Similarly, the ratio of brain weight to body weight increased further to $0.086 \pm .003$ and $0.086 \pm .004$ for G^+L^- and G^-L^- groups respectively. The concentrations of protein and DNA, although increased at 21 day, remained unaffected. The ratio of protein to DNA remained little elevated but was not statistically significant.

These results are expressed on the basis of " % of control" in Table 21. As it is evident from the same, the deficits in body and brain weight increased as the undernutrition progressed to 21 days. In association with those changes, the ratio of brain weight to body weight increased from 102 at birth to 268 % in G^-L^- group. However, the concentrations of protein and DNA did not suffer any significant reduction although the ratio of the same increased from 99 at birth to 105 % at 21 days in G^-L^- group.

The data also suggests some decrease in the size of these deficits at 21 days. This may be due to operation of an adaptive mechanism whereby brain growth and maturation are maintained at the expense of body growth. The ratio of brain weight to body weight was much greater in the undernourished animals, the differences between controls and undernourished animals becoming more evident with the progress of undernutrition (Table 21A).

The results of effects of undernutrition on the brain enzymes are presented in Table 20. The activities of AChE and ChAc increased with age. There was a four-fold increase in AChE and eleven-fold in ChAc at 21 day over the initial at birth. The values for AChE activity at various ages are in broad agreement with those of Adlard and Dobbing (1971a); Im et al. (1971a); Gambetti et al. (1972) and Bajgar et al. (1972). Similarly, the values for ChAc are similar to those of Ladinsky et al.(1972) and Eckhert et al. (1976a). However, the variation observed as compared to other reports is due to the differences in the methodology used. The method using radioactive Acetyl-CoA as a substrate is known to give higher values as compared to that of using radioactive acetate, as in the present case.

Table-20 : Effects of maternal protein deficiency during gestation and/or lactation on the brain cholinergic enzymes of the progeny at different ages.[1]

Age (days)	Dietary regimen	AChE (μmoles subs.hydrolysed/min. Brain g.	AChE Protein mg.	AChE DNA mg.	ChAc(μmoles ^{14}C-ACh formed/hour. Brain g.	ChAc Protein mg.	ChAc DNA mg.
7	G^+L^+ (4)[2]	3.56±0.29	0.961±0.005	1.8±0.14	0.68±0.06	0.012±0.001	0.33±0.01
	G^+L^- (4)	3.85±0.31	0.067±0.003	2.0±0.14	0.62±0.03	0.011±0.001	0.31±0.03
	G^-L^- (4)	2.49±0.20*	0.043±0.004*	1.3±0.13*	0.50±0.02*	0.008±0.003	0.26±0.01**
14	G^+L^+ (8)	4.8 ±0.20	0.064±0.004	2.2±0.07	1.88±0.04	0.025±0.001	0.87±0.02
	G^+L^- (8)	4.4 ±0.25	0.059±0.004	2.1±0.16	1.60±0.08**	0.021±0.001*	0.77±0.03
	G^-L^- (8)	3.7 ±0.04**	0.050±0.001*	1.8±0.06**	1.38±0.06**	0.018±0.001‡	0.68±0.03‡
21	G^+L^+ (6)	9.2 ±0.3	0.095±0.004	4.1±0.20	3.96±0.18	0.041±0.002	1.75±0.08
	G^+L^- (7)	10.4 ±0.2**	0.110±0.003**	4.9±0.12**	3.45±0.14**	0.036±0.002	1.62±0.07
	G^-L^- (5)	8.2 ±0.02*	0.086±0.001*	3.8±0.06	3.27±0.10**	0.035±0.001*	1.55±0.04*

Values are expressed as Mean ± S.E.M.

1. Dams were fed respective diet from the first day of conception or first day post-partus till 21 days after partus. G^+L^+ = 20% protein diet during gestation and lactation; G^+L^- = 5% protein diet during lactation; G^-L^- = 5% protein diet during gestation and lactation.

2. Number in parenthesis indicate number of observations.
Values marked with asterisks are significantly different from the control.
* ($p<0.05$) ; ** ($p<0.01$); ‡ ($p<0.001$).

Table- 21A Effects of maternal protein deficiency on the progeny at different ages.

Age (days)	Dietary regimen	Values as % of control values							
		Body wt.	Brain wt.	Brain wt. / Body wt.	Protein mg/g	DNA mg/g	Protein / DNA	AChE Activity/g	ChAc Activity/g
At Birth	G^-	72*	77*	102	99	101	99	85*	89
7	G^+L^-	59*	89*	162*	98	94	106	108	91
	G^-L^-	41*	70*	169*	99	93	104	70*	73*
14	G^+L^-	41*	74*	186*	98	96	102	92	85*
	G^-L^-	30*	58*	198*	97	95	102	77*	73*
21	G^+L^-	30*	80*	268*	98	94	104	113*	87*
	G^-L^-	27*	75*	268*	98	93	105	89*	83*

* Values marked with asterisk are significantly different from the control values.

The activity of AChE was not affected in $G^{+}L^{-}$ group on 7 and 14 days, but it was elevated at 21 days (+ 13 %). Interestingly, the activity when expressed as per mg protein or DNA still showed significant increase. This suggests that cholinergic system is relatively spared from the effects of neonatal undernutrition. The fact that activity is more even on the basis per unit of protein and DNA confirms the suggestion of increased density of synapses and neurons per unit area of the brain in undernourished rats during early period of life (Cragg, 1972). However, contrary to this suggestion, there are reports of dinimished density of synapses and synaptic thickening (Jones, 1976). Our findings are in agreement with those of Im et al.(1971), Adlard and Dobbing (1972a; 1972b), Eckhert et al. (1976[a,b]) and Tyzbir et al. (1977) (Table-22). Adlard and Dobbing (1972[b]) suggested following possible explanations for raised activity of the enzyme :-

a) Altered kinetic properties of the enzyme.

b) General increase in enzyme concentration within membranes.

c) A 'sparing' of structures in which the enzyme is present. However, since AChE is found mainly in high concentrations in nerve endings it is tempting to speculate that the raised levels of the enzyme represent a 'sparing' of nerve endings relative to

other cell fractions. For confirmation of such a view much more morphological work would have to be done (Jones, 1976).

As mentioned earlier, undernutrition during both gestation and lactation had more drastic effect on the brain development. As such AChE activity was found to be lowered even at birth in those pups delivered by mothers fed 5% protein diet from the first day of gestation. It is of interest to note, in Table 20, that AChE activity was in the deficit of 30, 23 and 11 % at 7, 14 and 21 days in $G^{-}L^{-}$ group. The deficits were showing a downward trend suggesting a possible 'catch up' with the normal activity. But even at 21 days it was still significantly lower than the normal. This is in agreement with the other reports on similar treatment for undernutrition (Adlard and Dobbing, 1971a; 1971b; Eckhert et al., 1976a for forebrain only). This would suggest that the 'sparing' observed in the case of neonatal undernutrition is not, possibly, applicable in this situation. This could be due to more severe degree of retardation in brain maturation. But the point made above about the possible "catch up" with age is supported by the elevation observed in the activity of brain AChE in those rats undernourished from gestation, lactation followed by either continued undernutrition or rehabilitation (Adlard and Dobbing, 1972; Im et al., 1972; Tyzbir et al., 1977).

ChAc activity showed a differential trend. At birth it was not found to be lower due to prenatal undernutrition. Even at day 7 it remained unaffected in $G^{+}L^{-}$ group. However, it was lowered at 14 and 21 days (the deficits being 11 and 13 %). In case of $G^{-}L^{-}$ group it was significantly lowered at 7, 14 and 21 days; the deficits being 26, 26 and 17 % respectively. In this case also the deficits were more in $G^{-}L^{-}$ group as compared to $G^{+}L^{-}$ group suggesting the possible adverse effect of drastic undernutrition resulting into brain retardation. These results are in agreement with those of Eckhert et al.(1976)[a,b] and also those of Ladinsky et al. (1972) who observed reduced ChAc activity in rats neonatally retarded by hypothyroidism induced by antithyroid drugs. However, it is hard to explain the differential effects of undernutrition on AChE and ChAc activities.

It would not be out of place to dwell a little upon the time sequence of present studies. These studies were initiated way back in 1972-73 and since then number of reports have been published particularly on undernutrition and AChE activity. At one stage it was felt that the study would be an exercise in futility because of various reports were being published at a steady rate. However, it was realised after a close look at the work of various people (Table-21B) that the entire theme of this problem lacks

concerted efforts to find out the effects of an individual components of cholinergic system, i.e. ACh, AChE and ChAc. In addition to this, the diets used for producing prenatal or postnatal undernutrition were not sufficiently low in protein level to bring about drastic retardation in the development of the brain (Table- 2[illegible]). The present studies have been rewarding in a sense that we could get the complete picture of cholinergic system in an unified manner.

In summary, these studies suggest :

1) Brain ACh levels are adversely affected with drastic undernutrition. The G^-L^- regimen had more adverse effect than G^+L^- regimen.

2) AChE activity was increased at 21 days in G^+L^- group suggesting possible 'sparing' of nerve endings. However, ChAc activity remained lower at 21 days.

3) AChE and ChAc activities were reduced in G^-L^- regimen. The latter showed a tendency towards 'catch up' at a later age.

4) However, it is hard to explain the correlation between ACh on one hand and enzymes on the other hand.

5) The differential effects on the activities of AChE and ChAc are difficult to explain at this stage.

Table-2[illegible]: Compilation of values for AChE and ChAc under various nutritional treatments from the published literature (not exhaustive)

Reference	Dietary regimen	Age (days)	% Change		Remarks
			AChE per g	ChAc per g	
Sereni et al. (1966)	G^+L^- (4 & 16 litter size)	21	No effect	NE	AChE activity was reduced at earlier age but levelled off at 21 days.
Im et al.(1971)	G^+L^- (12% protein diet)	21	No effect	NE	Dietary protein levels in the present studies were much lower (5%).
	$G^-L^-R^+$ (12 & 25% protein diet)	49	+24%	NE	
		266	+14%	NE	
Adlard & Dobbing (1971a)	G^-L^- (50% restriction of food intake)	21	-11%	NE	This differs from our method of feeding 5% protein diet during both periods.
Adlard & Dobbing (1971b)	As above	21	-12% (F+S)	NE	-do-
Gambetti et al. (1972)	G^-L^- (8% protein diet from 10th day of gestation)	12	No effect	No effect	Difference in % protein in diet and timing of undernutrition.
		24*	+39%(Synaptosom)	+41%(Synaptosom)	

Continued....

Table-2.6 (Continued..)

Reference	Dietary regimen	Age (days)		% Change AChE per g	% Change ChAc per g	Remarks
Eckhert et al. (1976a)	$G^-L^+R^+$ (7, 25% protein in the diet)	63	F:	-11%	NE	Differences in protein levels in the diet.
			Cb:	-20%	+18%	
			St:	NE	-08%	
	$G^+L^-R^+$ (25,12,25% protein in diet)	63	F:	+10%	NE	-do-
			Cb:	+12%	+15%	
			St:	NE	-77%	
Adlard & Dobbing (1972)[a,b]	$G^-L^-R^-$ (50% restriction)	84	F:	+16%	NE	Difference in dietary restriction.
			Cb:	+11%	NE	
			St:	+ 8%	NE	
	$G^-L^-R^+$ (50% restriction + ad lib.)	84	F:	+10%	NE	-do-
			Cb:	+10%	NE	
			St:	+13%	NE	
Tyzbir et al. (1977)	$G^-L^-R^-$ (8% protein)	42		+18%	NE	Difference in the dietary protein levels.
	$G^+L^+R^-$ (45% protein)	42		+10%	NE	

F = Forebrain; Cb = Cerebellum; St = Brain stem; Rest for whole brain: NE= Not Estimated.
G = Gestation; L = Lactation; R = Rehabilitation.
+ = Normal diet. - = Protein deficient diet or undernutrition.
* Activity measured in recovered synaptosomal fractions of the cerebral cortex.

4. Studies on the Effects of early undernutrition and subsequent Dietary Rehabilitation on Cholinergic System :

Since early undernutrition was found to result in significant changes in the levels of individual components of cholinergic system, it was thought worthwhile to study the effects of dietary rehabilitation on the possible 'reversal' of these changes. For this purpose, pups born of mothers fed low protein diet from first day of pregnancy (G^-L^-) and those from birth onwards (G^+L^-) were fed 20 % protein diet ad lib. from 22nd day onwards for next five weeks. The control group (G^+L^+) was similarly reared on 20% protein ad lib. diet.

The results of these studies are presented in Table 22. As can be seen from the same, the animals subjected to undernutrition in early life and subsequently rehabilitated on the high protein (20%) diet had not achieved a complete 'catch-up' with regard to body and brain weights. The rats undernourished during lactation and subsequently rehabilitated ($G^+L^-R^+$) had a body weight of 74 $\pm$ 1.4 g, the rats undernourished during gestation and lactation and then rehabilitated ($G^-L^-R^+$) registered a body weight of 65 $\pm$ 1.5 g. as against 134 $\pm$ 3.0 g. of the control group ($G^+L^+R^+$). The body weight deficits in the

Table-22 : Effects of dietary rehabilitation on brain ACh, AChE and ChAc activity of rats undernourished in early life [1].

	$G^+L^+R^+$	$G^+L^-R^+$	$G^-L^-R^+$
No. of rats	10	10	10
Initial body wt.(g) at 21 days.	45 ± 1.0	13 ± 1.2**	11 ± 0.8**
Final body wt. (g)	134 ± 3.0	74 ± 1.4**	65 ± 1.5**
Brain wt. (g)	1.510 ± .0042	1.35 ± 0.022**	1.29± 0.040**
Brain ACh (4) (µg/g)	2.4 ± 0.20	2.05 ± 0.30	1.95± 0.40
AChE/g (6)	9.10 ± 0.32	10.48 ± 0.34*	10.13± 0.30*
ChAc/g (6)	4.30 ± 0.15	3.85 ± 0.10*	3.72± 0.15*

Values are expressed as Mean ± S.E.M.

1. Undernourished pups were switched over to 20% protein diet ad lib. on day 22nd; fed the same for next 5 weeks.
2. Number in parenthesis indicate number of observations per group.
   Values marked with asterisk are significantly different from controls.
   * ($p < 0.05$); ** ($p < 0.01$).

first two groups work out to be 45 and 51 % as compared to the controls. The same groups had body weight deficits of 70 and 72 % at 21 days of age (i.e., prior to rehabilitation). Therefore, it is apparent that although the deficits were narrowed at the end of rehabilitation, the significant deficits persisted. However, in terms of increments the control group increased by 197 %, $G^{+}L^{-}R^{+}$ group by 469 % and $G^{-}L^{-}R^{+}$ by 490 %, suggesting an obvious attempt at 'catch-up' of the earlier deficits. A similar picture was also observed as regards to brain weights. The values for brain weights were : control, 1.510 $\pm$ .042 g; $G^{+}L^{-}R^{+}$, 1.35 $\pm$ 0.022 g and $G^{-}L^{-}R^{+}$ 1.29 $\pm$ 0.040 g with the respective deficits of 10 and 14%. The same deficits were of the tune of 19 and 24 % at 21 days of age. Similar observations with regards to body and brain weights have been reportedd by Adlard and Dobbing (1971a; 1971b; 1972), Telang (1975) and Nakhasi (1975).

It is interesting to note that brain ACh levels returned to normal at the end of rehabilitation. The values for the control group was 2.4 $\pm$ 0.20, for $G^{+}L^{-}R^{+}$, 2.05 $\pm$ 0.30 and for $G^{-}L^{-}R^{+}$ 1.95 $\pm$ 0.40 μg/g brain. Although the deficits were statistically not significant, these were 14 and 18 % in the last two groups respectively. However, at 21 days of age the corresponding deficits were 16 and 26 %. As mentioned earlier, the reduction observed in the levels of

other neurotransmitters in the undernourished rats was thought to be transitary (Sereni et al., 1966). The present results suggest perhaps similar transitary reduction which was subsequently reversed by the rehabilitation. However, it would be pertinent to point out that total ACh content in the whole brain would be significantly reduced in the rehabilitated group. The values for the same work out to be 3.6 µg for the control, 2.8 µg for $G^+L^-R^+$ group (27 % deficit) and 2.5 µg for $C^-L^-R^+$ group (31 % deficit). In view of these and earlier observations, brain ACh levels seem to be reduced in those conditions associated with drastic reduction in the body and brain weights. But this reduction seems to be transitary and is reversed to significant extent by dietary rehabilitation. However, the questions of adaptation to the diet and environmental stimulation vis-a-vis functional state of the brain and ACh levels would not be answered at this stage. For this purpose, more detailed studies should be carried out to throw more light on these aspects.

As expected, AChE activity remained elevated at the end of rehabilitation. The control group had an activity of 9.10 ± 0.32; $G^+L^-R^+$ group mustered 10.48 ± 0.34 ($p < .05$) and $G^-L^-R^+$ registered activity of 10.13 ± 0.30 ($p < .05$) µmoles of acetylthiocholine iodide hydrolyzed per minute per gram brain. The elevation in the two experimental groups was

of the order of 15 and 11 % respectively. These groups prior to rehabilitation showed elevation in AChE activity of 13 % in $G^{+}L^{-}$ and deficit of 11 % in $G^{-}L^{-}$ group at 21 days. In other words, rats undernourished during gestation and lactation had reduced AChE activity (-11 %) at 21 days of age but subsequently this activity "cross-overed" to positive side, finally registering a rise of 11 %. Whereas in the rats undernourished during lactation period alone AChE activity was higher at 21 days and even after rehabilitation remained elevated to the same extent.

It is possible that the elevated AChE activity represents a relative 'sparing' of regions rich in the enzyme, for example the basal ganglia. Despite the deficit in brain weight, rehabilitated animals had a whole brain AChE activity which was the same as that in the controls (Controls, 13.7, $G^{+}L^{-}R^{+}$; 14.1 and $G^{-}L^{-}R^{+}$ 13.1 μmoles substrate hydrolyzed min/brain).

This further supports the argument of 'sparing' of cholinergic system from undernutrition. Similar observations have been reported by other workers (Adlard and Dobbing, 1971a; 1971b; Adlard and Dobbing, 1972; Im et al., 1971: Gambetti et al., 1972; Eckehrt et al., 1976a; 1976b; Tyzbir et al., 1977; Coupin et al., 1977).

Contrary to the rise observed in AChE activity, ChAc activity was found to be reduced in both the undernourished groups. $G^+L^-R^+$ and $G^-L^-R^+$ groups registered the ChAc activities of 3.85 ± 0.10 and 3.72 ± 0.15 µmoles $^{-14}$C-ACh formed per hour per gram brain as against 4.3± 0.15 of $G^+L^+R^+$ group. The deficits work out to be 10 and 13 % respectively. Similar deficits at 21 dsys were 12 and 17 % respectively. There is some reduction in deficits after rehabilitation but still the activity was significantly lower than that in the controls. Eckhert et al. (1976a) reported decreased ChAc activities in brain stem and increased activity in cerebellum of rehabilitated rats after initial undernutrition during gestation and/or lactation period. Neonatal undernutrition followed by postweaning protein deficiency was also associated with similar deficit in ChAc activity in cerebellum but not in brain stem. In another report, Eckhert et al. (1976b) also reported significantly decreased ChAc activity in brain stem of $G^-L^-R^+$ rats. As mentioned earlier, Ladinsky et al. (1972) reported decreased ChAc activity in hypothyroidism induced by giving antithyroid drugs in rats.

In this connection, it is interesting to note that in rehabilitated rats various brain lipid fractions were found to be deficient. The fractions in deficits were cholesterol (Dobbing, 1968; Culley and Linenberger,1968;

and Geison and Waisman, 1970), phospholipids (Howard and Granoff, 1968; Culley and Linenberger, 1968) and galactolipids (Culley and Mertz, 1964; and Geison and Waisman, 1970). On the other hand, some studies suggest a complete disappearance of these deficits in the rehabilitated animals (Benton et al., 1966; Guthrie and Brown, 1968).

As mentioned earlier, the growth of axons and dendrites, establishment of synaptic junctions and increase in number of glial cells and processes take place in post-natal three weeks in rats (Davison and Dobbing, 1968 and Dobbing, 1971). All these processes are known to be affected by inadequate nutrition (Bass, 1970) which delays functional maturation and causes permanent histological and biochemical abnormalities (Culley and Lidenberg, 1968; Dobbing, 1968; Altman et al., 1971; Bass, 1971; Dobbing et al., 1971; Neville and Chase, 1971; Shoemaker and Wurtman, 1971; Smart and Dobbing, 1971a; 1971b). Malnutrition in the rats was also reported to diminish cortical synapses (Bass et al., 1970). However, Cragg (1972) reported that in rats undernourished during first 3 and 7 weeks of life there was a 22-33 % increase in the density of the neuronal cell bodies in the visual and frontal areas as compared to controls. But the presynaptic endings of the neuropil were not affected. Early undernutrition has been also reported

to affect number of dendritic processes (Salas et al., 1974), number of synaptic connections (Cragg, 1972) and thickness of pre- and post-synaptic densities (Jones and Dyson, 1976). The crucial question is whether any of these deficits and histological changes can be subsequently rectified ? As mentioned earlier, the deficits in body weight, brain weight, protein, and DNA content and lipids were not reversed. On the histological front, Dyson and Jones (1976) and Jones and Dyson (1976) found that synaptic organization was of less mature distribution in 16 weeks old rehabilitated rats. The synaptic organization was comparable to 3 week old control rats; but better than that of undernourished rats. This suggests limited extent of process of 'catch-up'.

The present results are in agreement with regards to body and brain weights and ChAc activities. Though ACh levels were normalised, AChE activity remained elevated. This suggests that individual components of cholinergic system seem to be affected in differential manner and these components respond to rehabilitation in different manners. Or otherwise these differences could be due to the different degree to which these components were affected in the various areas of the brain. Such a possibility has also been suggested by Eckhert et al. (1976a).

Another interesting aspect of early undernutrition is the irreversible effects observed on behaviour. The long lasting increases in brain AChE were found to parallel the long-term increases in the emotional reactivity observed in adult rats given identical nutritional treatment (Levitsky et al.,1970). Early undernutrition has also been associated with lasting effects on activity and social behaviour of rats (Whatson et al., 1976). Brain cholinergic system has been demonstrated to be involved in the control of behaviour (Russel, 1969). Moreover, the long term effects of early handling give similar results in that both emotional reactivity (Denenberg, 1962) and subcortical AChE activity decreased (Tapp & Markowitz 1965). Recently Im et al. (1976) reported that environmental isolation resulted in increased AChE specific activity in control rats whereas, decreased the same in rats undernourished during early period of life. It is interesting to note that behavioural abnormalities have been reported in children undernourished during early period of life (Cravioto et al., 1966; Chase and Martin, 1970; Chase and Metcalf, 1975).

5. Effects of Postweaning Undernutrition and Protein Deficiency :

As mentioned earlier, brain ACh concentration is thought to vary inversely with functional activity of the brain. It is known to be affected differentially by conditions like anaesthesia and narcosis on one hand, and electrical stimulation and emotional excitment and stress conditions on the other (Richter and Crossland, 1949; Crossland and Merrick, 1954; Elliot et al., 1950; Naik et al., 1970). Moreover, the concentration of ACh in the brain of the rat increases with age upto 100 days or more (Crossland, 1951; Naik et al., 1970). In view of above observations and since the adult brain is considered 'aplastic' it was felt worthwhile to study the effects of undernutrition and protein deficiency during post-weaning period on the cholinergic system.

As described earlier, weaning rats were reared either on 5, 8, 20 or 20 % restricted protein diets for a period of five weeks. The results of this experiment on brain ACh levels are presented in Table 23. As expected, body weights were significantly reduced in all the experimental groups as compared to that of 20 % protein diet ad lib. group. It is interesting to note that mean body

weight in 5% protein diet group was 66 ± 3.6 g and this was comparable with that of 20 % protein diet fed at 50 % level (Group No.5), 59.0± 2.7 g. The brain weights of animals reared on 5%, 8% protein and 20% protein diet restricted were significantly lower than that of the controls. However, 20% protein diet pair-fed to 5% protein diet did not affect the brain weight significantly.

ACh content of the brain was lowered in 5 %, 8 % and 20 % protein diet severely restricted groups. However, ACh concentration was affected significantly only in 5 % protein group and 20 % protein severely restricted (25 % of 5% protein food intake - Group No.6). In other words, both protein deficiency and very severe calorie restriction (Group 6) resulted in significantly lower amounts and concentrations of brain ACh, whereas with less severe degrees of food restriction only the total amount in the brain and not the concentration was affected. This was also true of the 8 % protein diet. This contrasts with the differential effects of calorie and protein deficiencies during postweaning period on brain enzymes (Rajalakshmi and Ramakrishnan, 1972). In these studies, protein deficiency but not the severe undernutrition resulted in lower activities of brain glutamate dehydrogenase and glutamate decarboxylase although body weight in the latter were much less than in former. It is, however, interesting to note

that although the retardation in body weight was comparable in the protein deficient and severely undernourished animals (Groups 1 and 5), the amount and concentration of brain ACh were significantly more in the latter case.

The finding of a lower brain ACh with severe undernutrition contrasts with the increase reported in starvation (Naik et al., 1970). This may be, as mentioned earlier, because the effects of chronic undernutrition are different from those of complete starvation.

Since brain ACh levels were affected in post-weaning protein and calorie deficient rats, the study was further extended to other parameters of the cholinergic system. The effects on the brain protein and DNA levels are presented in Table 24. As can be seen from the same, the reduction in body and brain weights was similar to those described earlier. The ratio of brain weight to body weight was found to be significantly elevated in protein deficient and calorie restricted animals. As expected brain protein and DNA levels were not found to be significantly altered. The ratio of protein to DNA, although little reduced, was not significantly altered. However, in terms of total content of protein and DNA in whole brain would be altered to the same tune as that of reduction in brain weights.

Table_24 : Effects of postweaning deficiencies of protein and calories on the body and brain weights , brain protein and DNA[1].

Group No.	Diet	Body wt. (g)	Brain wt. (g)	Brain wt. / Body wt.	Protein (mg/g)	DNA (mg/g)	Protein / DNA
1	5% Protein	63** (5) ±6.0	1.496* ±0.015	0.024* ±0.002	117.5 ±6.7	1.50 ±0.06	79.4 ±7.6
2	8% Protein	105 (5) ±7.0	1.418** ±0.090	0.014 ±0.004	111.1 ±0.38	1.40 ±0.05	79.7 ±2.3
3	20% Protein	133 (7) ±13.0	1.600 ±0.043	0.012 ±0.001	114.1 ±3.7	1.38 ±0.05	82.6 ±5.0
4	20% Protein (Pair-fed to 5% protein)	105 (7) ±6.0	1.502 ±0.046	0.015 ±0.001	116.9 ±4.4	1.50 ±0.03	78.6 ±3.7
5.	20% Protein (Restricted)[2]	64** (7) ±7.0	1.435* ±0.030	0.024** ±0.002	119.2 ±1.6	1.52 ±0.05	78.0 ±3.3
6.	20% Protein (Severely restricted)[3]	45** (8) ±2.3	1.357** ±0.029	0.030** ±0.001	113.3 ±6.5	1.44 ±0.04	76.1 ±4.1

Values are expressed as Mean ± S.E.M.

1. Weaning rats were fed on the respective diet for 5 weeks.
2. 50% of the ad lib. food intake of animals fed 5% protein diet.
3. 25% of the ad lib. food intake of animals fed 5 % protein diet.

* ($p < 0.05$); ** ($p < 0.01$).

The data on the activities of brain enzymes is presented in Table 25. It is evident that the activities of AChE and ChAc were not affected by severe protein deficiency or calorie restriction. However, Eckhert et al. (1976a) reported differential effects of post-weaning undernutrition on these two enzymes in the brain regions. Activity of AChE was found to be increased in forebrain and brain-stem whereas activity of ChAc was increased in cerebellum and decreased in brain stem. Unfortunately, the activity of AChE in cerebellum and activity of ChAc in forebrain of rats fed protein deficient diet after weaning periods were not studied. The lack of any effect of protein deficiency on the brain enzymes contrasts with the observation of decrease in brain glutamate decarboxylase and dehydrogenase in this condition (Rajalakshmi and Ramakrishnan, 1972). None the less, neither protein deficiency nor post weaning undernutrition was found to have any effect on the concentrations of cholesterol and phospholipids in the brain (Dobbing and Widdowson, 1965; Dickerson et al.,1972 and Nakhasi, 1975).

In view of these findings, weaning rats were further continued on the protein deficient diet for a period of 65 weeks to assess the possible effects on the brain enzymes. The results of the same are presented in Table 26.

Table-25: Effects of postweaning deficiencies of protein and calories on the brain enzymes[1].

Group No.	Diet	AChE (μmoles sub. hydrolysed per minute.			ChAc (μmoles ^{14}C-ACh formed per hour		
		g Brain	mg protein	mg DNA	g Brain	mg Protein	mg DNA
1	5% Protein (5)	9.04 ±0.53	0.077 ±0.004	6.09 ±0.52	4.64 ±0.21	0.039 ±0.003	3.11 ±0.32
2	8% protein (5)	9.92 ±0.39	0.092 ±0.010	6.95 ±0.43	4.45 ±0.28	0.041 ±0.005	3.12 ±0.27
3	20% Protein (7)	9.12 ±0.73	0.081 ±0.008	7.25 ±0.71	4.58 ±0.23	0.040 ±0.003	3.62 ±0.24
4	20% Protein (7) (Pair-fed to 5% Protein)	9.25 ±0.49	0.080 ±0.006	6.18 ±0.23	4.53 ±0.20	0.039 ±0.003	3.03 ±0.12
5.	20% Protein (7) (Restricted)[2]	9.02 ±0.50	0.077 ±0.007	5.92 ±0.33	4.29 ±0.30	0.037 ±0.003	2.81 ±0.14
6	20% Protein (8) Severely restricted[3]	9.34 ±0.43	0.084 ±0.006	6.30 ±0.41	4.13 ±0.21	0.037 ±0.003	2.78 ±0.19

Values are expressed as Mean ± S.E.M.

1. Weaning rats were fed on the respective diet for 5 to 6 weeks.
2. 50% of the *ad lib.* food intake of animals fed 5% protein diet.
3. 25% of the *ad lib.* food intake of animals fed 5% protein diet.

Table-26 : Effects of extended post-weaning protein deficiency on the brain enzymes[1].

Diet	20% Protein	5% Protein
1. Body weight (g)	247 ± 30	146 ± 15*
2. Brain weight (g)	1.736 ± 0.039	1.521 ± 0.041*
3. Brain weight / Body weight	0.007 ± 0.001	0.011 ± 0.001*
4. Brain		
Protein (mg/g)	124.9 ± 1.9	126.1 ± 2.3
DNA (mg/g)	1.87 ± 0.09	1.87 ± 0.08
Protein / DNA	67.2 ± 3.9	68.4 ± 4.2
5. AChE (µmoles substrate hydrolyzed/min.)		
g. brain	7.15 ± 0.32	7.32 ± 0.65
mg.protein	0.057 ± 0.003	0.058 ± 0.005
mg.DNA	3.8 ± 0.1	3.9 ± 0.4
6. ChAc (µmoles ^{14}C-ACh formed/hr)		
Brain (g)	4.46 ± 0.18	4.60 ± 0.22
Protein (mg)	0.035 ± 0.001	0.037 ± 0.002
DNA (mg)	2.41 ± 0.21	2.52 ± 0.15

Values are expressed as Mean ± S.E.M.

1. Weaning rats were fed respective diets for 65 weeks ad lib.

* ($p < 0.05$).

The extended protein deficiency was associated with significant deficits in body and brain weights. However, the activities of AChE and ChAc remained unaffected.

As mentioned earlier, it is hard to explain lack of any correlation between ACh levels and the activities of AChE and ChAc. Significant decrease in a metabolite, ACh, without any apparent alterations in the activities of synthesising and breaking down enzymes need further detailed studies. One of the major aspects could be the environmental stimulation and handling of the animals (Eckhert et al., 1975a; Levitsky et al., 1970; Denenberg, 1964). Secondly, the possibility of changes in the activities of the enzymes, in some way, may not be directly correlated with ACh concentration which is very sensitive to stress conditions and various drug treatments. This possibility is partly justified considering that very short time was required for ACh levels to return to normal after initial fall due to electric stimulation (Richter and Crossland, 1949). However, it would be too hasty to draw any conclusion of this sort until further studies throw more light on these aspects of cholinergic system.

In summary, the postweaning undernutrition and protein deficiency resulted in significant deficits in brain

ACh levels without any apparent alterations in the activities of the cholinergic enzymes. If the changes observed in ACh level are functional, it would mean that cholinergic system remains vulnerable even at adult age.

6. Effects of Pre- and Post-natal Thiamine Deficiency :

As mentioned earlier, thiamine deficiency has been implicated to interfere with the cholinergic system. There have been numerous reports of reduction in brain ACh levels in thiamine deficient animals. However, there are no reports on the effects of deficiency on developing central nervous system, particularly with regards to cholinergic system. Hence, it was thought worthwhile to study these aspects at different stages of brain maturation.

For this purpose, thiamine deficiency was induced by feeding mothers thiamine deficient diet (TD) from 7th day of gestation and the controls were pair-fed similarly (PFC). In the earlier experiments, mothers fed thiamine deficient diet from first day of pregnancy were found to result in large number of fetal resorption and miscarriages. Therefore, the deficient diet was introduced from 7th day of gestation onwards and continued till weaning age.

Reproductive performances of TD and PFC females are presented in Table 27. Since the deficiency was instituted from 7th day of gestation there was no question of comparison with regard to % fertility *per se* . Average litter size was not affected. However, number of still births in TD females was higher. Similarly mortality was higher (17%) in TD group as compared to PFC group (6 %). Average birth weight was not much reduced. It is interesting to note that TD females had more bleeding during the partus and generally partus lasted for a longer time. Similar observations have been recorded much earlier by Stähler in 1937. In some cases, TD females killed pups and consumed them. The body weight at 21 days of age was much reduced with a deficit of 25%. Similar deficits in body weights have been reported by Trostler *et al.*(1977). After 21 days of age, pups were further reared, individually, on the deficient diet.

The pups were killed on 7, 14, 21 and 28 days of age for the study on the individual cholinergic components. The results of this study on brain ACh levels are presented in Table 28. As can be seen from the same, maternal thiamine deficiency during gestation and lactation resulted in retarded body weight gain from 14th day onwards. The body weights at 28 days were 27$\pm$ 2.7 g for PFC group and

Table- 27 : Effects of thiamine deficient diet on reproductive performance.[1]

	Dietary regimen	
	Control Pair-fed	Thiamine deficient
1. No. of females kept for breeding	30	42
2. No. of pregnancies	26	35
3. % Fertility	86	83
4. Average litter size	8	8
5. No. of pups still born	3	40
6. No. of live births	205	240
7. No. of deaths in neonatal period	12	40
8. % Mortality	6	17
9. Average birth weight (g)	6	5.5
10. Average weaning weight (g)	24	18

1. Dams were fed thiamine deficient diet from 7th day of gestation, controls were pair-fed to the deficient group. All pups were not utilized for experiments.

Table- 28 : Effects of pre- and post-natal thiamine deficiency on brain ACh levels.[1]

Age (days)	Dietary regimen	Body weight[3] I (g)	Brain weight II (g)	ACh (µg) III Brain	ACh (µg) IV per g brain
7	PFC	8 ± 0.4	0.525±0.015	0.75±0.05	1.43±0.04 (4)[2]
	TD	7 ± 0.4	0.498±0.012	0.71±0.06	1.41±0.08 (4)
14	PFC	13 ± 0.8	0.832±0.027	1.26±0.12	1.48±0.12 (4)
	TD	10 ± 1.0*	0.746±0.032	1.02±0.14	1.35±0.13 (4)
21	PFC	20 ± 2.4	1.175±0.040	1.79±0.17	1.51±0.12 (4)
	TD	16 ± 0.9**	1.093±0.032	1.25±0.17*	1.15±0.09*(4)
28	PFC	27 ± 2.7	1.275±0.049	2.16±0.23	1.68±0.12 (4)
	TD	21 ± 1.7*	1.152±0.062	1.20±0.16*	1.03±0.08** (4)

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 7th day of conception till weaning, thereafter pups were individually fed. The controls were pair-fed (PFC) similarly.
2. Number in parenthesis indicate number of observations. Each group consisted of 8 pups per dam.
3. Values are rounded off to the nearest whole figure.

* ($p<0.05$); ** ($p<0.01$).

21 ± 1.7 g for TD group, the deficit being 25 %. However, brain weights were not adversely affected. This suggests that the deficiency restricts more of body weight than brain weight. However, the restriction of food intake in PFC group must have contributed considerably to the retardation in body and brain weight. Trostler et al. (1977) observed similar retardation in PFC group as compared to ad lib. control. The comparison with our data on ad lib. control (Table 19) reveals that the body weight deficit works out to be 55 % at 21 days. As against this, the deficit in brain weight was only 3%. This substantiates our contention that food restriction affects more of body weight than brain weight.

In this connection it is interesting to note that Trostler and Sklan (1977) reported that percentage of thiamine transfer from mother to pups was reduced after 14th day post-partus. Although milk composition of thiamine deficient dams (G^-L^-) at 18 days post-partus revealed similar protein and amino acids levels as in controls, milk protein fractions showed some differences. In the deficient animals, milk lactose levels were reduced, whereas fatty acid content was increased.

Brain ACh content and concentration remained unaffected till 14 days of age. The deficits in content

Table-30 : Effects of pre- and post-natal thiamine deficiency on brain enzymes.[1]

Age (days)	Dietary regimen	AChE (μmoles substrate hydrolysed per minute			ChAc (μmoles ^{14}C-ACh formed per hour		
		g brain	mg protein	mg DNA	g brain	mg Protein	mg DNA
7	PFC (4)[2]	3.46±0.35	0.057±.008	1.71±.25	0.60±.05	0.010±.001	0.29±.03
	TD (4)[2]	3.58±0.17	0.059±.003	1.84±.12	0.62±.05	0.010±.003	0.32±.02
14	PFC (4)	5.31±0.60	0.070±.004	2.49±.21	1.72±.12	0.023±.004	0.81±.03
	TD (4)	4.87±0.41	0.067±.003	2.32±.07	1.54±.11	0.021±.001	0.73±.05
21	PFC (4)	6.75±0.29	0.082±.004	3.22±.14	3.45±.07	0.043±.003	1.56±.09
	TD (4)	6.04±0.21	0.076±.002	2.73±.06	3.15±1.4	0.039±.0005	1.42±.01
28	PFC (4)	8.40± 0.17	0.097±.001	3.83±.11	3.61±.14	0.041±.001	1.64±.02
	TD (4)	7.97±0.26	0.095±.001	4.04±.06	3.40±.22	0.041±.002	1.72±.05

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 7th day of conception till weaning, thereafter pups were individually fed. The controls were pair fed (PFC) similarly.

2. Number in parenthesis indicate number of observations. Each group consisted of 8 pups per dam.

However, the comparison of these values with ad lib. group in Table 19 suggests a significant reduction in the activities of both enzymes.

In other words, maternal thiamine deficiency during gestation and lactation resulted in significant deficits in brain ACh concentration and content at 21 and 28 days of age without affecting the activities of cholinergic enzymes. The discrepancy in the observed deficit in ACh without any alterations in the activities of AChE and ChAc is discussed elsewhere, at length.

Since the activities of both the enzymes were not affected in the whole brain of the deficient rats it was thought worthwhile to study the effects on the regions of the brain. The results of these studies are presented in Table 31. It can be seen that weights of cortex and cerebellum were reduced in TD group as compared to PFC group. However, the weight of medulla + pons + stem remained essentially unaffected. The brain protein levels in all the regions remained unaffected. However, DNA level was significantly reduced ($p < 0.05$) in medulla + pons + stem region of the deficient group. Similarly, the activity of AChE was also significantly reduced in this region as compared to PFC group. These results are hard to explain

Table-31 : Effects of pre- and post-natal thiamine deficiency on regional AChE activity in the brain of rats at 21 days of age.

Dietary regimen	Body weight (g)	Brain region	Wet wt. (g)	Protein (mg/g)	DNA (mg/g)	AchE µmoles/ min/g.
PFC	23±0.5(16)[2]	Cortex	0.950 (16) ±.008	80.3(4) ±3.4	1.62(4) ±0.04	1.985(4) ±0.196
		Cerebellum	0.150(16) ±.004	87.5(4) ±1.5	6.35(4) ±0.20	1.652(4) ±0.226
		Medulla & Pons +Stem	0.115 ±.003	97.0(4) ±7.5	1.93(4) ±0.02	3.665(4) ±0.297
TD	18±0.4(16)[2]	Cortex	0.871(6)** ±.007	85.7(4) ±3.4	1.63(4) ±0.02	2.482(4) ±0.276
		Cerebellum	0.134(16)* ±.004	86.9(4) ±2.8	6.38(4) ±0.26	1.737(4) ±0.292
		Medulla & Pons Stem	0.108(16) ±.003	88.1(4) ±1.5	1.69(4)* ±0.06	2.518(4)* ±0.339

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 7th day of gestation onwards. The controls were pair-fed (PFC).
2. Numbers in parenthesis indicate number of observations.

* ($p<0.05$); ** ($p<0.01$).

as the whole brain values, seen earlier, remain unaffected in the deficient group. However, it is interesting to note that increase in brain DNA and protein levels as compared to ad lib. controls have been reported in the whole brain of thiamine deficient rats (Dreyfus, 1976a). In another report, the levels of phospholipids, cerebrocides and cholesterol were found to be reduced in thiamine deficient rats as compared to pair fed controls (Trostler et al., 1977). Another interesting aspect of this observation was that histopathological lesions of thiamine deficiency were selectively located at brain stem and lateral vestibular nucleus (Dreyfus, 1976b). These regions are known to be rich in thiamine phosphates and specially vulnerable to thiamine deficiency. The highest depletion is seen in these regions. However, any relation between these observations and the presently found reduction in AChE activity in medulla + pons + stems, at this juncture, cannot be elucidated.

7. Effects of Neonatal Thiamine Deficiency:

Since maternal thiamine deficiency during gestation and lactation was found to result in significant deficits in brain ACh levels in the pups at 21 and 28 days of life, it was thought worthwhile to study the effects of the deficiency during lactation period alone on the cholinergic system.

For this purpose, dams delivered on the same day were selected, pups in the litter size of 8 were randomly allotted to each dam. One group was fed thiamine deficient diet ad lib. from first day of lactation. The controls were pair-fed to the deficient animals. From day 21 onwards, pups were individually fed. The pups were killed at 7, 14, 21 and 28 days for the study.

The results on brain ACh levels in the deficient animals at different ages are presented in Table 32. The body weights were significantly lowered in the deficient animals from 14 days onwards. The deficits in body weights were 20, 36 and 36 % at 14, 21 and 28 days of age respectively. However, the values of body weights were more in comparison to those of pups born of mothers fed the deficient diet during gestation and lactation (Table- 28). The brain weight was not significantly reduced at any of the ages, the observation in agreement with that of earlier one (Table-28). However, brain ACh content and concentration were significantly reduced only at 28 days of age. The deficits were 25 and 24 % as compared to the pair-fed controls. The symptoms of thiamine deficiency involving neurological dysfunction were apparent towards the end of 4th week. It is interesting to recall that these symptoms appeared in the middle of third week in the case of pups born of mothers fed the deficient diet throughout gestation

Table-32 : Effects of post-natal thiamine deficiency on brain ACh levels[1].

Age (Days)	Dietary regimen	Body weight[3] (g)	Brain weight II (g)	ACh (µg) III Brain	ACh (µg) IV per g. Brain
1	-	5 ± 0.09	0.210± 0.010	0.14 ± 0.04	0.63 ± 0.02 (4)[2]
7	PFC	9 ± 0.50	0.553± 0.014	0.83 ± 0.03	1.50 ± 0.05 (4)
	TD	9 ± 0.40	0.545± 0.014	0.81 ± 0.04	1.49 ± 0.04 (4)
14	PFC	15 ± 0.90	0.877± 0.022	1.41 ± 0.14	1.58 ± 0.10 (4)
	TD	12 ± 1.00*	0.838± 0.014	1.28 ± 0.05	1.52 ± 0.08 (4)
21	PFC	39 ± 1.30	1.310± 0.016	2.23 ± 0.07	1.70 ± 0.04 (8)
	TD	25 ± 1.50**	1.240± 0.016	1.90 ± 0.18	1.53 ± 0.10 (8)
28	PFC	59 ± 1.2	1.432± 0.016	2.48 ± 0.11	1.73 ± 0.09 (8)
	TD	38 ± 1.5**	1.364± 0.008	2.01 ± 0.05**	1.47 ± 0.03** (8)

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 1st day postpartum and controls (PFC) were pair-fed with food intake of TD group. The deficient group showed typical symptoms of neurological dysfunctions towards the end of 4th week.
2. Number in parenthesis indicate number of observations. Each group consisted of 8 pups per dam. Two brain samples were pooled in some cases.
3. Values are rounded off to nearest whole figure.

* ($p < 0.05$); ** ($p < 0.01$).

and lactation, this was also associated with reduced levels of ACh at 21 days. This suggests that maternal thiamine deficiency during both the periods precipitated more of a thiamine depletion in the milk as compared to the deficiency during lactation alone. The progressive depletion was more in the former group as can be seen from Table- 28 as compared to the latter group (Table- 32). This was also obvious from the enhanced deficit in ACh level (39 %) in the former group as compared to the latter one (24 %).

The results of postnatal thiamine deficiency on brain protein and DNA levels are presented in Table 33. The body weights were similarly affected as in earlier experiment. The brain weights, as earlier, remained unaffected. Brain protein and DNA levels were not significantly affected at any age. However, brain protein levels showed a general increase in thiamine deficient rats. Similar observations have also been made by Dreyfus (1976a). The data on the activities of AChE and ChAc are recorded on Table-34. As expected, the activities of both the enzymes remained unaffected in the deficient group. However, the activities of both enzymes were higher in pair-fed and the deficient group as compared to those in the pups born of the mothers fed the deficient diet during both gestation and lactation (Table- 30).

Table-33 : Effects of post-natal thiamine deficiency on body weight, brain weight, brain protein and DNA levels.[1]

Age (days)	Dietary regimen		Body wt (g)	Brain wt. (g)	Brain wt. / Body wt.	Protein (mg)	DNA (mg/g)	Protein / DNA
At Birth		(6)[2]	5.0±0.09	0.240±.010	0.047±.008	49.1±2.0	1.90±.04	25.9± 1.3
7	PFC	(6)	8.0±0.30	0.542±.015	0.068±.001	65.8±3.3	2.21±.10	29.9± 1.1
	TD	(8)	7.5±0.40	0.541±.017	0.074±.006	62.4±3.8	2.24±.11	27.8± 1.1
14	PFC	(8)	14.0±0.90	0.867±.019	0.062±.003	76.3±2.1	2.21±.07	34.6± 0.07
	TD	(8)	11.0±0.90*	0.836±.014	0.075±.007	73.0±2.4	2.29±.11	32.7± 0.80
21	PFC	(8)	38.0±0.10	1.294±.018	0.034±.001	88.1±3.4	2.22±.10	40.0± 2.1
	TD	(8)	23.0±0.90**	1.240±.015	0.053±.001**	83.6±2.0	2.15±.08	38.6± 0.9
28	PFC	(8)	58.0±0.60	1.446±.012	0.025±.003	89.2±1.2	2.13±.06	39.8± 1.6
	TD	(8)	38.0±0.90**	1.40 ±.010	0.037±.0008**	92.0±2.2	2.12±.09	44.5± 2.8

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 1st day post-partum and controls (PFC) were pair-fed with food intake of TD group. The deficient group showed typical symptoms of neurological dysfunction towards the end of 4th week.

2. Number in parenthesis indicate number of observations. Each group consisted of 8 pups per dam.

* ($p < 0.05$); ** ($p < 0.01$).

Table- 34: Effects of postnatal thiamine deficiency on brain enzymes.[1]

Age (days)	Dietary regimen		AChE (μmoles substrate hydrolyzed per minute)			ChAc (μmoles ^{14}C-ACh formed per hour)		
			g Brain	mg Protein	mg DNA	g Brain	mg Protein	mg DNA
At Birth	-	(5)[2]	2.86±.07	0.060±.004	1.48±.04	0.37±.02	0.0076±.0007	0.190±.015
7	PFC	(6)	3.52±.24	0.054±.004	1.60±.12	0.65±.05	0.009 ±.001	0.300±.030
	TD	(8)	3.80±.26	0.063±.003	1.74±.11	0.63±.04	0.009 ±.001	0.28 ±.02
14	PFC	(8)	5.18±.36	0.067±.003	2.37±.13	1.71±.08	0.022 ±.0004	0.776± .031
	TD		4.94±.24	0.067±.002	2.19±.04	1.56±.07	0.021 ±.001	0.697±.033
21	PFC	(4)	6.89±.36	0.076±.008	3.11±.22	3.46±.07	0.038 ±.003	1.560±.07
	TD	(4)	6.34±.16	0.072±.002	2.69±.08	3.34±.11	0.038 ±.001	1.41 ±.07
28	PFC	(4)	9.15±.25	0.102±.003	4.30±.25	3.79±.17	0.042 ±.002	1.78 ±.13
	TD	(4)	8.71±.16	0.097±.004	4.55±.38	3.68±.14	0.040 ±.004	1.92 ±.14

Values are expressed as Mean ± S.E.M.

1. Dams were fed thiamine deficient diet (TD) from 1st day post-partum and control (PFC) were pair-fed with food intake of TD group. The deficient group showed typical symptoms of neurological dysfunction towards the end of 4th week.

2. Number in parenthesis indicate number of observations. Each group consisted of 8 pups/dam.

In summary, maternal thiamine deficiency during lactation seems to affect body weight and not the brain weight. ACh content and concentration were reduced only at 28 days. However, the enzyme activities remained unaffected.

The values for body and brain weight and components of cholinergic system of the two modes of maternal thiamine deficiency as compared to their respective pair-fed controls are presented in Table-35. From the same, a comparison can be made between the two modes of the deficiency. The deficits in the body weights were in both the groups. This was apparent as the controls were pair-fed with the deficient group. However, from third week onwards the pups started consuming the diet which resulted in alteration in the body weight profiles. The deficits in the brain weights were more, although not significantly, in the pups born of mothers deficient during gestation and lactation ($B_1G^-L^-$) as compared to $B_1G^+L^-$ group. However, these deficits were statistically not significant. The values for protein levels were also comparable at all ages except at 28 days where the level was higher in $B_1G^+L^-$ group. Although the alterations in DNA levels were statistically not significant, the values for $B_1G^-L^-$ group were on the lower side.

The comparison between ACh concentration in the two groups is interesting. As can be seen from Table-35 and

Table-35 : Effects of maternal thiamine deficiency during gestation and/or lactation on the cholinergic system of the progeny at different ages.

Age (Days)	Dietary regimen	Values as % of control values						
		Body wt.	Brain wt.	Protein (mg/g)	DNA (mg/g)	ACh (µg/g)	AChE (Activity/g)	ChAc (Activity/g)
7	$B_1G^+L^-$	94	99	95	101	99	107	97
	$B_1G^-L^-$	94	96	98	94	98	103	103
14	$B_1G^+L^-$	78*	96	96	108	96	95	91
	$B_1G^-L^-$	64*	89	96	90	92	92	90
21	$B_1G^+L^-$	64*	95	95	97	94	92	96
	$B_1G^-L^-$	75*	90	95	94	76*	90	91
28	$B_1G^+L^-$	65*	96	103	99	76*	95	97
	$B_1G^-L^-$	70*	95	97	90	61*	95	94

Values marked with asterisks differed significantly from the control.

also from Figure-6, brain ACh levels were consistently lower in $B_1G^-L^-$ groups as compared to $B_1G^+L^-$ groups. The deficits were significantly more in the former group at 28 days whereas, in the latter at both 21 and 28 days. As discussed earlier, the depletion of thiamine is expected to be more in $B_1G^-L^-$ group as compared to $B_1G^+L^-$ group. This would contribute to lower levels of ACh observed in the former group at earlier age. Secondly, the typical symptoms of thiamine deficiency were seen earlier in the former group.

However, the activities of AChE and ChAc were not found to be significantly affected throughout the course of thiamine deficiency. The similar observation has been made by Heinrich et al. (1973) in the thiamine deficient adult rats in which brain ACh levels were significantly reduced. The discussion on the possible effect of thiamine deficiency on the activity of pyruvate dehydrogenase complex which in turn would affect availability of acetyl-CoA for ACh synthesis are discussed in the following portion of this Chapter.

From the foregoing observations, it is obvious that maternal thiamine deficiency per se during gestation and/or lactation does not seem to have any drastic effect on the brain maturation. However, the resultant decrease

Table-36 : Effects of pre- and post-natal thiamine deficiency and subsequent rehabilitation on brain ACh levels.[1]

G r o u p	Control	Rehabilitated
Initial Body wt. (g)	25.000 ± 2.3	20.000 ± 1.3
Final body wt. (g)	124.000 ± 5.6	114.000 ± 9.4
Brain wt. (g)	1.500 ± 0.030	1.440 ± .035
ACh (µg)		
Whole brain	3.37 ± 0.37	2.95 ± 0.20
g. brain	2.20 ± 0.21	2.02 ± 0.13

Values are expressed as Mean ± S.E.M.

1. Pups reared on thiamine deficient diet upto 28 days were switched over to normal diet for further 5 weeks. Pair-fed controls were similarly fed on ad lib diet.

rehabilitated deficient group. The deficit in the body weight was 8 %. This suggests that the deficit in the body weights was narrowed after rehabilitation. The brain weights were also comparable in both the groups.

The deficit in the brain ACh level at 28 days was of the order of 39 %. Whereas, at the end of rehabilitation period ACh levels had returned to normal. The respective ACh levels in the control and the rehabilitated group were 2.2 ± 0.21 and 2.02 ± 0.13 µg per g brain. There was no significant difference in the brain ACh content also. As mentioned earlier, the reduction in the ACh levels was thought to be transitory. In the undernourished rats, brain ACh levels returned to the normal after dietary rehabilitation. This seems to be also applicable in the present case. It is also interesting to note that the symptoms of thiamine deficiency disappeared within a week of starting the rehabilitation diet.

Recently, Trostler et al.(1977) reported that the dietary rehabilitation of the thiamine deficient pups resulted in the reversal of earlier changes in the phospholipids and cholesterol levels in the brain. However, the brain cerebrocide levels remained unaffected after

rehabilitation. In another study, administration of pyrithiamine to new born rats, during the critical period of myelogenesis, resulted in the decreased activities of brain transketolase and pyruvate decarboxylase; Whereas, myelination, as indicated by biochemical and morphological criteria, proceeded at an essentially normal rate (McCandless et al., 1976). These workers suggested that tentative metabolic pathways may be operational in the newborn rat brain enabling it to circumvent major blockage in thiamine-dependent reactions.

9. Postweaning Thiamine Deficiency and Brain ACh Levels :

As mentioned earlier, thiamine deficiency has been associated with neurological dysfunctions involving, perhaps, cholinergic system. Numerous studies have been reported to affect ACh levels in the deficient animals (Gubler, 1968; Cheney et al., 1969 and Heinrich et al. 1973). Contrary reports have also been recorded (Stern and Igic, 1970; Speeg et al., 1970; Vorhees et al., 1977). The logic behind the possible reduction has been the reduced availability of acetyl-CoA with reduced activity of PDH.

With the strong arguments, based on the findings, for and against the possible effects of the deficiency on brain ACh level in view, we approached this problem in a

different manner. Prior to entering into the details of this problem, the following aspects of the deficiency have been investigated :-

a) Onset and progress of thiamine deficiency with detailed observations of appearance of neurological symptoms;

b) The extent of reduction in thiamine levels of liver, heart and the brain regions of the symptomatic deficient rats; and

c) The possible effect on the moisture content of these tissues, possibly, resulting into artifact which might be introduced in expressing the results on the basis of wet weight.

The design of the experiment, as mentioned earlier consisted of feeding weaning rats on different levels of thiamine. Briefly the groups were as follows :

Group No.	Thiamine levels	% B_1	B_1/1000 calories
1.	1.5 mg/kg diet fed ad lib.	100	0.5
2.	1.5 mg/kg diet pair-fed to group 6	100	0.5
3.	0.75 mg/kg diet pair-fed to group 6	50	0.25
4.	0.375 mg/kg diet pair-fed to group 6	25	0.125

contd..

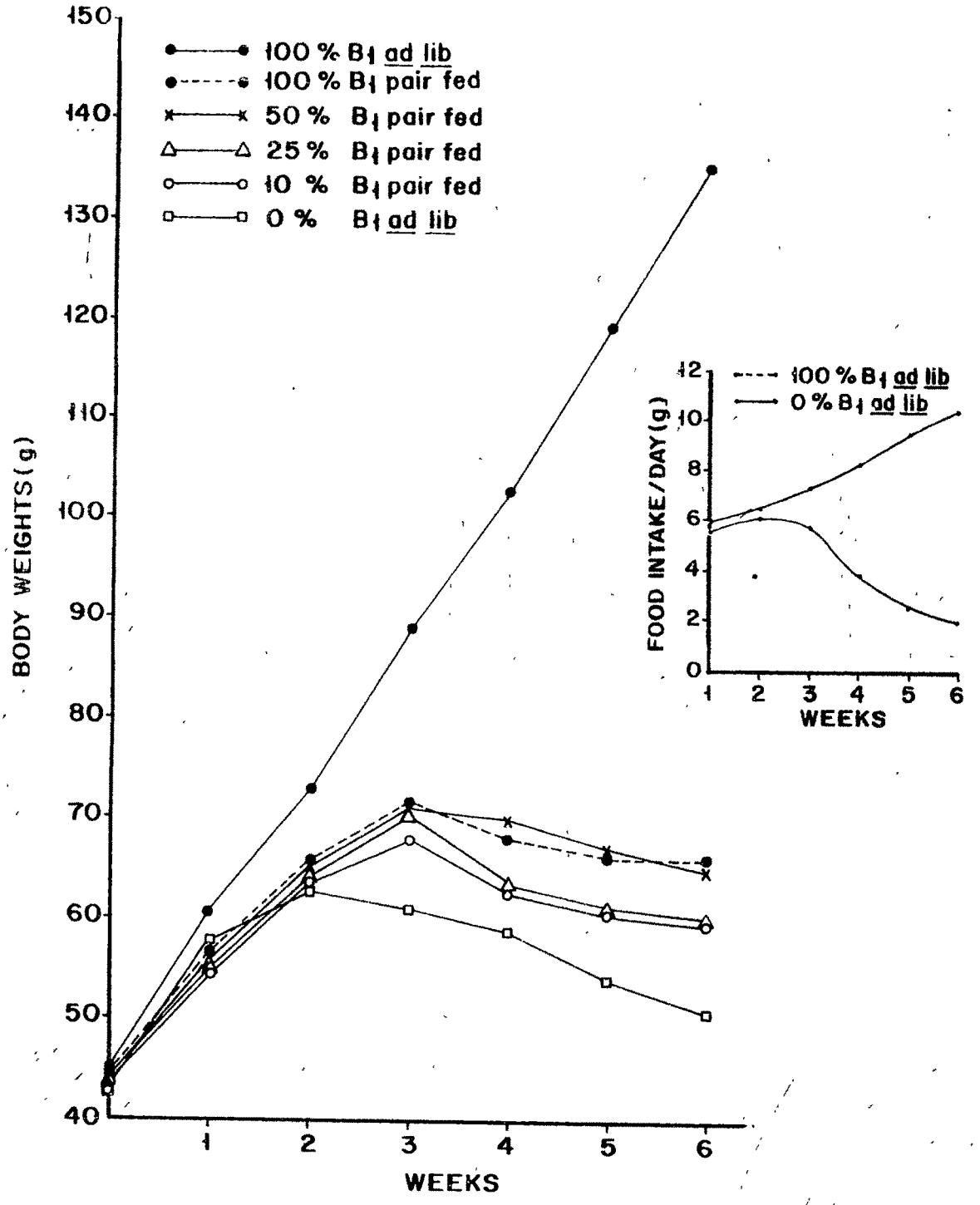

Fig. 7 :- Body weight gains of weaning rats fed different levels of thiamine (B_1) Each point represents mean of eight values. Pair-fed groups were pair-fed to the food intake of 0 % B_1 group

Inset represents the food intake per day of the two ad lib groups

the extent of anorexia present in the deficient group.

As against the deficit of 80 % in food intake, the reduction in body weight gain as compared to 100 % B_1 *ad lib.* works out to be approximately 94 %. However, this reduction as compared to 100 % B_1 pair-fed group works out to be 76 %. The comparison of the reductions in body weight as compared to 100 % B_1 *ad lib.* and pair-fed groups indicates that the contribution to this deficit due to anorexia and thiamine deficiency *per se*.

It is of interest to note that the profiles of body weight gains in 100 % and 50 % B_1 pair-fed groups are similar. This is borne out by the fact that the final mean body weights in these two groups were 66 $\pm$ 2.6 g (66-77 g) and 65 $\pm$ 1.7 g (60-70 g) respectively. This gives rise to an interesting query : Why this similarity exists inspite of 50 % reduction in thiamine intake? As pointed out earlier, the thiamine concentrations in these two groups were 0.5 and 0.25 mg per 1000 calories. The fact that thiamine requirement is proportional to calorie intake is very well known. In view of the requirement of thiamine being 0.27 to 0.33 mg per 1000 calories (Ziporin *et al.*, 1965) it is obvious that 50 % B_1 level, as can be seen, could still take care of the basic requirement of

thiamine by the pair-fed rats. Hence the latter group could muster similar weight gain.

However, the further reduction in dietary thiamine level to 25 % resulted in more deficit in the body weight. The final mean body weight was 61 ± 1.4 g (55-66 g). The deficit was around 6 % in comparison to 100 % B_1 pair-fed group. Interestingly, the rats pair-fed 10 % B_1 diet showed similar deficit of 10% with mean body weight of 59 ± 1.9 g (54-61 g). In other words, the levels of thiamine at 25 % and 10% had almost similar effect on body weight gain indicating, perhaps, that the B_1 concentrations of 0.125 and 0.05 mg per 1000 calories had a comparable capacity to maintain the weight gain.

It is of importance to note that the thiamine deficient group (0 % B_1) gained 21 g of body weight at the end of second week but thereafter lost the weight reaching the value of 51 ± 1.8 g (40-57 g). The initial gain could be attributed to body stores of thiamine which were depleted by the end of second week. Furthermore, the deficiency advanced quite rapidly resulting into 80 % deficit in food intake and 94% deficit in the body weight gain in comparison to 100% B_1 *ad lib.* group.

McCandless et al.(1968) induced dietary thiamine deficiency by feeding B_1 deficient diet consisting 18 % protein, 10 % oil and 68 % sucrose. At the end of 35 days regimen, the controls had final body weights of approximately 200 g as compared to 80 g and 52 g of pair-fed and the thiamine deficient rats (extrapolated from the graph). The initial body weights as seen from their graph varied between 55-60 g. The deficits in the body weights of the deficient animals as compared to the two controls were 74% and 35%. These deficits in our investigation were 62% and 22% at the end of 6 weeks. The differences could be attributed to higher initial body weights and the difference in the diet composition used by these workers. However, the profiles of body weight gains in both the studies were comparable. Gubler (1976a) reported the body weight gains of 100 g and 20 g in ad lib. control and thiamine deficient group at the end of 20-25 days (as extrapolated from the graph). Whereas the weight gains in the present investigation were 100 g and 18 g at the end of 3-4 weeks, which are in broad agreement with that of Gubler's (1976a) values as extrapolated from the published graphs. However, Vorhees et al. (1977) reported the weight gains of approximately 140, 38 g and negligible in the ad lib. control, pair-fed control and the deficient groups respectively at the end of 5th week. The initial body weight and the diet composition, however, were

different from the present investigation. Although, the true comparison in strict sense, can not be made in between all these studies, the fact remains that the general trend in the body weight gains remained essentially same. This is not to deny the fact that the weight gains observed in our studies were somewhat different which could be additionally attributed to the stock of the animals, animal house conditions and climatic variations.

During the course of progressive thiamine deficiency, careful observations revealed a typical sequence of appearance of symptoms of thiamine deficiency. For this purpose our observations were restricted to 0 % B_1 *ad lib.* group. Until the end of second week, while the rats were gaining weights, there was no appearance of symptoms of neurological dysfunction. At this stage, in few rats, some hair loss was seen. However, from the third week onwards the symptoms were clearly seen. The sequence of the appearance paralleled the decline in food intake and body weight gain. Towards the end of third week, the deficient rats developed incoordination in walking, impaired righting reflex and reluctance to walk. As the deficiency progressed the rats started to exhibit more of typical symptoms like walking in circular motion, imbalanced locomotion and loss of righting reflex. At the terminal stage, the animals had an appearance of dehydrated and shrivelled body with a great

reluctance to make any movement and persistent drowsiness.

The appearance of neurological symptoms, observed at the end of third to fourth week, coincided with the fall of body weight below 58-60 g. The frank symptomatic stage was reached at 55-58 g of body weight. The similar sequence of progressive appearance of symptoms have been reported by McCandless et al. (1968). Although the actual body weight values of the control and deficient rats were different in their studies, the appearance of neurological symptoms started around the same body weights (55-60 g). However, these workers observed this stage at 4-5 weeks as compared to 3-4 weeks in our studies. This must be purely because it took longer time to deplete thiamine stores in the deficient rats with initial body weights of 55-60 g in the reported studies. The similar pattern of growth profiles and appearance of neurological symptoms have been recorded by Dreyfus (1961). It is needless to mention at this stage that these symptoms, described above, were the result of CNS dysfunction and not of peripheral neuropathy (Church, 1935).

It would not be out of place to summarise the above results and the conclusions thereof. In the first instance, the body growth patterns observed in our experiments

were comparable with the reported values. The symptomatic rats showed the reported sequence of appearance of typical neurological symptoms. Moreover, the mortality rate was much higher at this stage of deficiency. In other words, we made sure that the deficient rats used for further studies had all the characteristic symptoms of thiamine deficiency.

Besides the manifestation of above symptoms the biochemical criterion to establish the degree of thiamine deficiency was thought to be essential. For this purpose studies were carried out to estimate total thiamine levels in the tissues of the deficient rats. The choice of this parameter was mainly based on the observation that the total thiamine levels truely reflect the concentrations of thiamine pyrophosphate and thiamine triphosphate in the brain of the deficient animals (Gubler, 1976b).

The results of these studies are presented in Table 37. The liver thiamine levels in the ad lib. control and pair-fed controls were 8.44 $\pm$ 1.21 and 10.3 $\pm$ 1.5 µg/g. Although the level in the latter group was higher it was not statistically significant. However, this elevation could be due to underutilization of thiamine stores in the liver due to restriction of calorie intake. The value for

liver thiamine level in the deficient group was much lower (2.35 ± 0.2 µg/g)and significantly different as compared to both the controls. The deficits in liver thiamine levels as compared to the two controls were 72 and 77 % respectively. The extent of reduction observed is similar to the reported deficits in liver thiamine values of pigeons treated with thiamine antagonists (Steyn-Parve, 1967).

The thiamine levels in the heart of the two controls and the deficient group were 10.2 ± 1.7, 12.7 ± 0.31 and 3.48 ± 0.89 ug/g respectively. As in the case of liver thiamine levels, the pair-fed controls had more of thiamine in the heart as compared to ad lib. control. The deficits in heart thiamine levels in the deficient animals were 65 and 72 % as compared to ad lib. and pair-fed controls respectively. As mentioned earlier, the thiamine deficiency has been known to result into bradycardia and cardiomegaly with the decrease in heart rate and reduced activity of certain enzymes. The observed changes in heart thiamine levels are, perhaps, indicative of the development of such a condition.

The thiamine levels in the different regions of the brain are also presented in Table- 37. The levels

Table-37 : Effects of post-weaning thiamine deficiency on the levels of total thiamine in liver, heart and the brain regions

(µg/g tissue or region)

G r o u p	Liver	Heart	Brain Regions		
			Cortex	Cerebellum	Medulla+Pons + Stem
1. Control, ad lib.	8.44(8) ± 1.21	10.2 (8) ± 1.7	3.02 (4) ± 0.11	3.56 (4) ± 0.46	5.13 (4) ± 0.65
2. Control, Pair-fed	10.30 (8) ± 1.50	12.7 (8) ± 1.2	2.76 (4) ± 0.31	2.41 (4) ± 0.23	3.48 (4) ± 0.61
3. Thiamine-deficient	2.35[a,b] (8) ± 0.21	3.48[a,b] (8) ± 0.89	0.93[a,b] (4) ± 0.16	0.71[a,b] (4) ± 0.16	1.69[a,b] (4) ± 0.25

Values are expressed as Mean ± S.E.M.

No. of observations are shown in parenthesis.

(a) Significantly different from group No.I ($p<0.01$).

(b) Significantly different from group No.II ($p<0.01$).

were decreased in all the brain regions studied. The reduction in the levels, however, was different in the different regions. The thiamine concentration in cortex of the ad lib. control, the pair-fed control and the deficient group were 3.02 ± 0.11, 2.76 ± 0.31 and 0.93 ± 0.16 µg/g respectively. The deficits in the last group work out to be 69 % and 66 % as compared to both the controls respectively. The cerebellum B_1 levels were 3.56 ± 0.46, 2.41 ± 0.23 and 0.71 ± 0.16 in the above three groups and the respective deficits being 80 % and 70 % respectively. In the pooled regions (medulla + pons + stem) the levels were 5.13 ± 0.65, 3.48 ± 0.61 and 1.69 ± 0.25 µg/g, respectively with the deficits of 67 % and 51 %. The computed values for the whole brain content of thiamine works out to be 4.9, 4.2 and 1.3 µg in the respective three groups with a deficit of 70 % in the deficient group. Whereas, the computed concentrations in the respective groups work out to be 3.3, 2.7 and 0.9 µg/g brain with a 66 % deficit in the deficient group.

Dreyfus (1959) reported the distribution of thiamine in the different regions of the brain. The B_1 concentration was in the following order : vermis, 21.1; medulla, 13.4; hypothalamus, 13.0; cortex, 13.0 µg/g of dry weight. Secondly he had also pointed out the large

variation in the values reported for thiamine concentration in the rat brain and liver. The reported values, as cited by him, were in the range of 2.2 - 6.0 µg/g fresh weight of the brain and 2.8 - 9.0 µg/g fresh weight of the liver. In view of these reports, the present values were in comfortable margin. However, the liver B_1 levels could be considered little on the higher side but this could be attributed to numerous factors like the stock of rats, diet and method of estimation. Moreover, the point which we are making is the degree of deficits in the deficient rats rather than the absolute values !

The distribution of thiamine levels in the present studies works out to be cortex, 25%; cerebellum, 30%; and the pooled regions, 45%. This pattern seems to be in agreement, by and large, with that reported by Dreyfus (1959). However, the pooled regions pose a problem of interpretation in terms of the levels in the individual region. Nevertheless, the computed contribution of these three regions to total thiamine of the whole brain works out to be around 16 % and in terms of concentration these regions are known to be equally rich in thiamine. Therefore, the thiamine levels of pooled regions as whole is expected to reflect, to large extent, the levels in individual regions. It is of interest to note that monophosphate and diphosphate esters of thiamine

make up about 80 % of the total thiamine in brain; the triphosphate ester and free thiamine each constitute approximately 10 % of the total thiamine in the brain (Dreyfus and Geel, 1976). However, the loss of the diphosphate is greater in the pons, the site of major pathological changes, than it is in any other part (Pincus and Grove, 1970).

This brings us to the observed deficits, as presented earlier, in the brain regions in the present study. McCandless et al. (1968) reported the deficits in thiamine levels in the whole brain of progressively deficient rats, the deficits at the end of $3\frac{1}{2}$ and $4\frac{1}{2}$ to 5 weeks were 73 % and 84 %. The symptomatic stage was around $4\frac{1}{2}$ weeks. However, in the reversal studies, a normal neurological state occurred with the brain thiamine levels rising to only 26 % of normal. Whereas, we have observed the computed deficit of 70 % in the whole brain B_1 levels at the end of 4.5 weeks in the symptomatic rats ; the split up of deficit was - cortex, 66%; cerebellum 71% and the pooled regions of medulla + pons + stem, 51 %. This would seem to be anomalous in view of the observation that total brain thiamine level in the symptomatic rats was less than 20 % of normal (Dreyfus, 1961; Balaghi and Pearson, 1966; Dreyfus, 1976b). Whereas, Hosein et al.(1966)

reported that neurological symptoms occurred only when the brain content of thiamine was lowered to 25 % of the normal values. This report was further supported by the work of others (DeCaro et al., 1956a; 1956b; Cerecedo and Eich, 1955). However, Gubler (1976b) reported, as adjudged from the published histogram, 65-70 % deficits in symptomatic rats at the end of 25 days of thiamine deficient regimen. Not only the symptomatic stage was reached at the deficits of ~~80-85~~ 65-70 % but decreased in glutamate level and increase in ∝-ketoglutarate levels in both blood and brain alone, with a significant decrease in the brain GABA levels were observed by Gubler et al. (1974). In other words, these workers could find symptomatic as well as biochemical changes in the brain of rats with deficits of 65-70 % in brain thiamine levels. Moreover, Church (1935) had also suggested that subtle neurological changes may occur with a somewhat lesser depletion of thiamine than that of 80 %. In view of this, the present deficits reported above could be considered to reflect the frank thiamine deficiency with the dietary regimen employed in this investigation.

However, the extent of reduction observed in the brain regions was variable. The highest reduction (72%) observed in cerebellum, is in agreement with the reportedly higher incorporation of radioactive thiamine in cerebellum

followed by the brain stem of deficient pigeon (Itokawa and Fujiwara, 1976). Nevertheless, as admitted earlier, the estimation of thiamine levels in the individual regions of medulla + pons + stem would have thrown more light on the actual pattern of B_1 depletion in these sensitive regions.

The question that remains is whether the observed decrease in thiamine levels in the thiamine deficient animals is functional ? The results presented above indicate clearly two major aspects of this question. One is the establishment of frank symptoms of neurological dysfunction at the terminal stages of the deficiency. Secondly, the body weight deficits observed were comparable with the reported values. Moreover, the mortality at this stage of deficiency was relatively higher (6 out of 14 rats died). The only point that stands out in resolving this question is the computed deficit of 70 % of total thiamine level in the whole brain. In light of the reports cited earlier, this deficit, in our opinion, fairly reflects the functional status of the animals. It goes without saying that more detailed studies are required to explain the variation in B_1 deficits in the different regions of the brain.

Ultrastructural changes in the brain stem of thiamine deficient rats have been studied by Collins (1967), Robertson et al. (1968) and Tellez and Terry (1968). Collins (1967) reported the cytoplasmic and nuclear swelling of glial cells prior to any other abnormalities. Robertson et al. (1968) also showed that the earliest lesions were associated with intracellular oedema involving glial cells and perivascular glial foot processes. Later, oedema involved the extracellular space and myelin sheaths. The impairment of energy-dependent electrolyte transport by the perivascular glia was thought to cause the oedema. Dreyfus (1976b) also pointed out that the oedematous changes taking place in the selective structures of the brain of progressively thiamine deficient animals. In view of the various reports on oedematous changes it was thought worthwhile to look into this aspect and ascertain whether such changes occur in a magnitude which could affect the moisture content of the whole brain and thereby introduce an artifact in expressing the biochemical parameters on the basis of fresh wet weight or per gram wet weight.

The results of this study are shown in Table 38. As can be seen from the Table, thiamine deficiency had no effect on the moisture content of any of the tissues studied. The average moisture content of the brain regions in the

control group was 78.69 ± 0.51 in cortex, 77.10 ± 0.13 in cerebellum and 72.56 ± 0.60 % in the pooled regions. Similarly, in the deficient group the values were 77.19 ± 0.30, 77.44 ± 0.79 and 72.22 ± 1.08 % respectively. Thus, moisture content of all the tissues studied remained essentially unaffected. The reported edematous changes taking place in finer structures of the deficient animals, in our opinion, require more sensitive histochemical methods to reflect such a change and the present methodology used, in no way, can confirm these reports. As said earlier, our objectives were limited only to gross changes in moisture content. And hence, from the results obtained it can be safely concluded that the expression of various biochemical parameters on the basis of fresh wet weight does not seem to involve the artifact due to gross changes in moisture contents of the tissues studied. However, the study of subcellular fractions and ultra structures may reveal more clear picture.

As can be seen from Table 39, the brain weights of the deficient groups were not significantly affected as compared to 100 % B_1 pair-fed controls. However, in the last group the brain weight showed a somewhat downward trend. This would naturally give rise to a question of whether or not the brain weight would have the same relationship to

Table- 38: Effects of post-weaning thiamine deficiency on moisture content of the tissue.

(Percent of fresh weight)

G r o u p	Liver	Heart	Brain regions		
			Cortex	Cerebellum	Medulla+Pons + Stem
1. Control, ad lib. (8)	69.16 ± 0.09	76.40 ± 0.27	78.69 ± 0.51	77.10 ± 0.13	72.56 ± 0.60
2. Control, Pair-fed (8)	68.44 ± 0.82	75.84 ± 0.08	78.17 ± 0.13	76.92 ± 0.76	70.59 ± 1.40
3. Thiamine-deficient (8)	69.72 ± 0.30	77.40 ± 1.40	77.90 ± 0.30	77.44 ± 0.79	72.22 ± 1.08

Values are expressed as Mean ± S.E.M.

No. of observations are shown in parenthesis.

body weight regardless of whether the body weight was attained by uninterrupted growth, or was attained after loss from a higher weight attained earlier, as in the case of the deficient rats. When the body weights were plotted against the brain weight per 100 g body weight for the control rats, used throughout the course of this investigation, the values for deficient rats fall essentially on the same curve as the control rats (Fig. 8). Thus, it appears that rats which attain a certain body weight and then lose weight during thiamine deficiency, also lose a proportionate amount of brain weight so that the brain weight/body weight ratio is the same for rats of a given weight, regardless of the direction from which the weight is attained.

Table 39, also shows the ACh content and concentration in the brain of the deficient rats. The 100 % B_1 pair-fed group had the brain ACh content of 2.54 $\pm$ 0.15 ug and concentration of 1.97 $\pm$ 0.12 µg/brain. The levels in the 50 % B_1, P.F. were significantly affected with a deficit of 21 %. As earlier mentioned, the body weight of this group was not affected in comparison to pair-fed group. In the next group (25 % pair-fed), ACh content and concentration were also significantly reduced with the deficit of 32 %. The point of interest is that the there was not

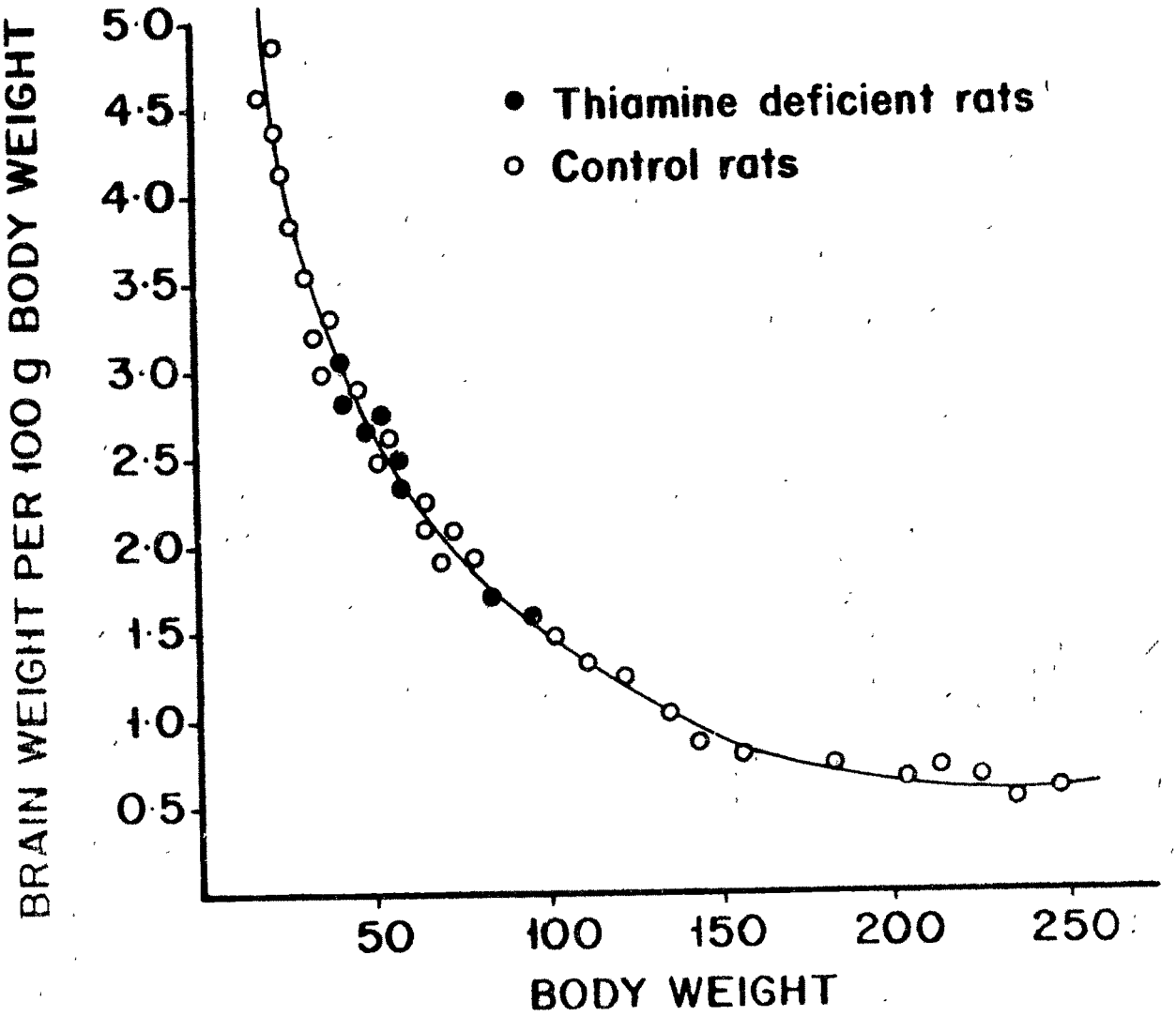

Fig.8 - Effect of age and thiamine deficiency on the relationship between brain weight and body weight in rats

Table-39 : Effects of post-weaning thiamine deficiency on brain ACh levels.[1]

Group No.		Dietary regiman	Brain wt. (g)	Acetylcholine (µg)		Acetylcholine (µg/g) as % of Group 2
				Whole brain	g. Brain	
1	(5)[2]	100% B_1 ad lib.	1.48±0.06	3.67±0.44	2.48±0.37	-
2	(6)[2]	100% B_1 Pair-fed	1.30±0.04[a]	2.54±0.15	1.97±0.12	100
3	(7)[2]	50% B_1 Pair-fed	1.31±0.03[a]	2.04±0.18[a]	1.56±0.13[a,b]	79
4	(7)[2]	25% B_1 Pair-fed	1.30±0.05[a]	1.73±0.16[a*b*]	1.35±0.10[a,b*]	68
5	(7)[2]	10% B_1 Pair-fed	1.31±0.04[a]	1.87±0.21[a*b*]	1.42±0.12[a,b*]	71
6.	(4)	0% B_1 ad. lib.	1.21±0.07[a]	1.22±0.22[a*b*]	0.97±0.18[a*b*]	49

Values are expressed as Mean ± S.E.M.

1. The rats were sacrificed at the end of 5-6 weeks, following the establishment of neurological symptoms.
2. Numbers in parenthesis indicate number of observations in the group.

a = Values significantly different from that of 100% B_1 ad lib. control ($p < 0.05$).
b = Values significantly different from that of 100% B_1 pair-fed control ($p < 0.05$).
a* = Values significantly different from that of 100 B_1 ad lib. control ($p < 0.01$).
b* = Values significantly different from that of 100% B_1 pair-fed control ($p < 0.01$).

much difference in the body weight of this group (61 ± 1.49) as compared to 50 % B_1 pair-fed group (65 ± 1.7). Nonetheless, brain ACh leve was significantly lowered ($p<0.01$). However, this group did not show any neurological symptoms at the end of experiment. In other words, the deficit of 32% in the brain ACh level was not of functional importance. The deficit in the ACh level remained almost same (29 %) in the 10 % B_1 pair-fed group. The similarity in the deficits of the latter two groups confirm the similarity in their average body weights (61 ± 1.4 g and 59 ± 1.9 g respectively), at the end of the experiment. This similarity, once again, confirm that these two levels of dietary thiamine could maintain similar thiamine status of the rats in the two groups. However, further depletion with time could have resulted in more drastic reduction in the body weight as well as brain ACh levels. The 0 % B_1 *ad lib.*, as mentioned earlier, had a body weight reduction of 74 % and 35 % as compared to the two controls. This group had brain ACh content of 1.22 ± 0.12 ug and concentration of 0.97 ± 0.18. These two values were significantly lower as compared to pair-fed control (p 0.01). The deficit in ACh concentration was 51 % which was drastically lower than the earlier groups. This reduced concentration reflects the true ACh level in the symptomatic rats.

Another interesting aspect of this study is the correlation between dietary thiamine levels and brain ACh content and concentrations. As shown in Figure 9, the decrease in dietary thiamine levels seems to result in the progressive deficits in brain ACh content as well as concentration.

As cited earlier, Cheney et al. (1969) reported the deficits of 28-30 % in brain ACh levels of thiamine deficient rats at the end of 25-30 days of regimen inspite of the fact that there was no indication of neurological disturbances. The similar deficits were also obtained following the treatment with pyrithiamine and oxythiamine, regardless of absence of any neurological symptoms in the rats received the latter antagonist. However, the incorporation of (3-^{14}C)-pyruvate, in vivo, into the quantity of ACh and total radioactivity in the brain of pyrithiamine treated rats was significantly increased. But similar increase was not observed in other two groups. On the other hand, the injections of eserine, given prior to development of convulsions, prolonged the life span of polyneuritic rats suggesting that the changes in ACh might play some role in the sequence of neurological events leading to death. The comparison between these results and those of ours reveal that the ACh deficit of 28-30 %

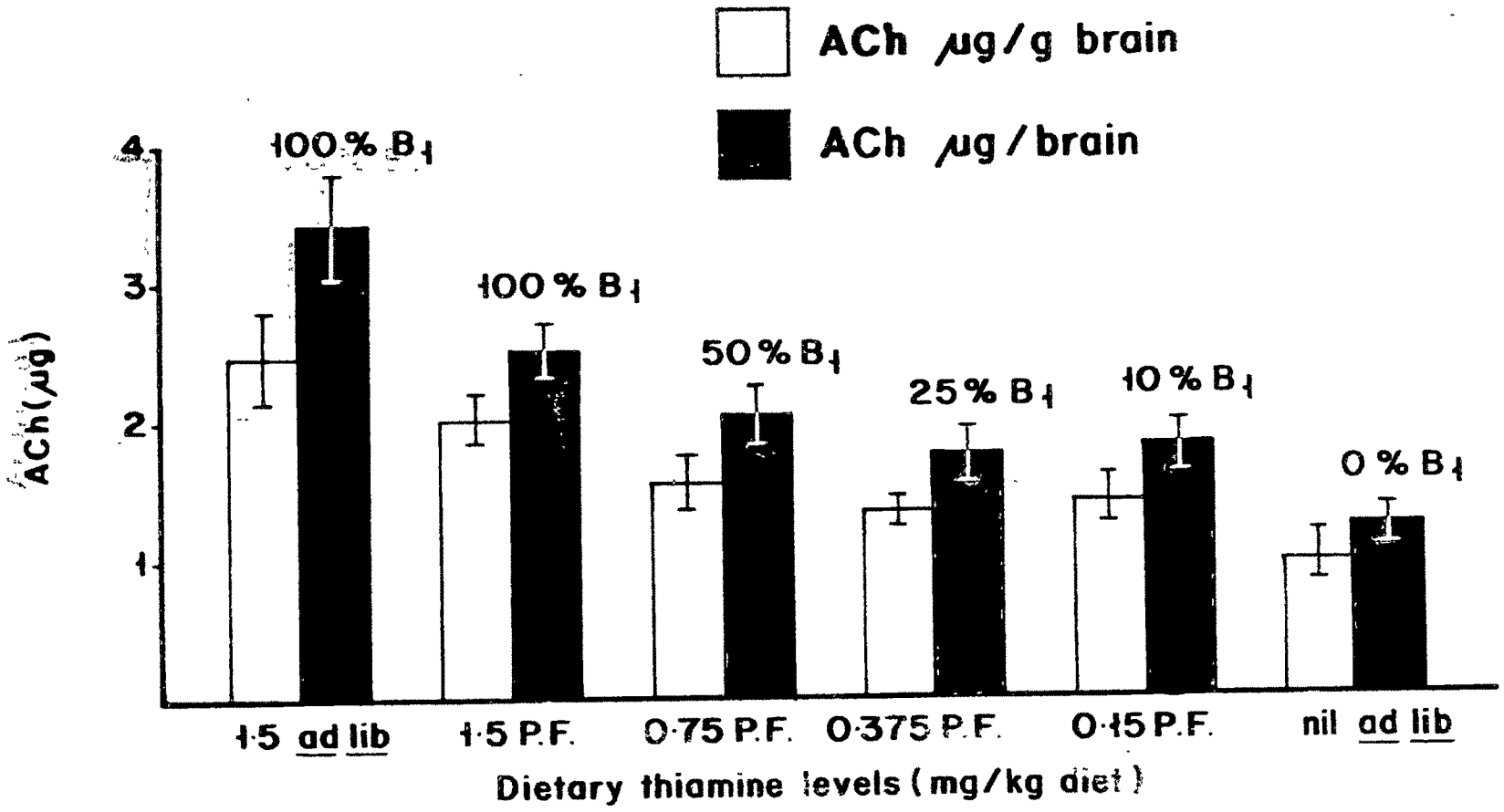

Fig 9 :- Brain ACh levels of the rats fed different levels of thiamine. Values represent mean of eight values with S.E. At the top of histogram thiamine levels as % of 1·5 mg/kg diet (control) are shown.
PF = Pair fed.

observed by these workers was comparable with the similar deficits of 32 and 29 % in 25 % B_1 and 10 % B_1 pair-fed groups, respectively. Secondly, these two groups were not found to exhibit the neurological symptoms which is in agreement with the above cited studies. In terms of absolute values our values for ACh are somewhat lower than those of Cheney et al. (1969). This may be due to the fact that their animals were given eserine, immediately before decapitation, while we did not follow this method in our studies.

As early as 1943, Lissak et al. reported a 37 % reduction in the concentrations of ACh in the brain and spinal cord of the thiamine deprived rats. Gubler (1968) also reported decrease in brain ACh levels of the deficient rats. Heinrich et al. (1973) observed the deficits of 42 % and 35 % in the concentrations of acetyl-CoA and ACh in the brain of the deficient animals. However, none of the enzymes of cholinergic system were affected. The reduction in the ACh concentration was correlated to decreased availability of the precursor, acetyl-CoA.

On the other hand, there have been reports that the thiamine deficiency had no effect on brain ACh levels in pigeons (Hosein et al. ,1966; Stern and Igic, 1970) and

in rats (Hosein et al., 1966; Speeg et al., 1970; Reynolds and Blass, 1975; Vorhees et al., 1977). Vorhees et al. (1977) carried out detailed studies on ACh levels and ACh utilization in the different areas of the rat brain. They failed to find any difference in the ACh levels of the deficient rats. However, ACh utilization was found to be decreased by 10-41 % in cortex, midbrain, diencephalon and brain stem of the deficient rats.

In the light of the above cited reports, for and against the effects of thiamine deficiency on the brain ACh levels, our results favour the earlier reports in favour of the adverse effect of the deficiency. Moreover, the present results indicate a fairly good correlation between the dietary thiamine levels and brain ACh concentrations. Soon after the completion of our studies Vorhees et al. (1977) reported their results (discussed earlier). These workers pointed out the possibility that the improvement in the techniques of killing the rats by microwave irradiation and analysis of ACh by a specific gas liquid chromatography would contribute much to the refinement in this type of investigation. However, these techniques are relatively recent ones and although elegant are out of reach for most of the workers. Moreover, one is inclined to think that the appropriate controls used

in the so far conventional techniques would reflect the same trend if not in absolute terms as judged by the new techniques.

Let us now examine critically various theories put forward for impaired synthesis of ACh in thiamine deficiency. First, it was suggested that a reduction in ACh synthesis could be induced in thiamine deficiency by reduction in the availability of high energy phosphates produced by TCA cycle. To substantiate this theory one would expect a reduction in the concentrations of various high energy phosphates in the brain of the deficient rats. Contrary, to this expectation, the content and turnover of brain ATP, ADP and AMP in the brain stem and cortex were found to be not affected by thiamine deficiency (McCandless et al., 1976; McCandless and Cassidy, 1976). A second theory being pursued to explain the decreased brain ACh levels was the reduction in the availability of acetyl CoA for brain ACh synthesis in the deficient rats. As mentioned earlier, Heinrich et al. (1973) reported 42 % deficit in brain acetyl-CoA levels of the deficient rats. However, Pyruvate dehydrogenase activity was not found to be affected. Reynolds and Blass (1975) failed to find any decrease in the brain acetyl-CoA levels of the deficient rats. Unfortunately, both the studies did not go into various regions of the brain. Thus, the question of reduced

availability of the precursor, acetyl-CoA, remains still unressolved. However, another line of evidence suggests an involvement of pyruvate metabolism in ACh synthesis. Gibson et al.(1975) reported that although the amount of ^{14}C-pyruvate ultimately leading to ACh constitutes less than 1 % of that resulting in $^{14}CO_2$ production, pyruvate dehydrogenase inhibition causes a measureable impairment in ACh synthesis. However, this does not explain the mechanism by which the pyruvate metabolism would bring about the change in ACh synthesis. It is pertinent to point out at this stage that rat brain ChAc has a Km of 18 μM for acetyl-CoA (White and Wu, 1973); whereas, acetyl-CoA levels range from 7 to 11 μM (Sollenberg, 1970; Shea and Aprison, 1975). In view of this, acetyl-CoA concentration seems to be conspicuously less than the Km values of the enzyme. This would mean that any further depletion in the acetyl-CoA level would adversely affect the rate of ACh synthesis as it happens in the case of choline levels (Fernstorm, 1977). Secondly, thiamine deficiency is known to cause the lesions in specific brain regions rich in thiamine diphosphate (Dreyfus, 1976b). In view of this one can not rule out all together, a possibility of finding reduced acetyl-CoA levels in these regions.

The reduced availability of acetyl-CoA in the deficient animals is thought to be mainly due to the reduced activity of pyruvate dehydrogenase (Heinrich et al., 1973). However, the reports on the alterations in the activities of this enzyme in the deficient animals have been contradictory. Koeppe et al. (1964) and Gubler (1968) did not find a change of enzymatic activity in brain homogenates from thiamine deficient animals. However, Dreyfus and Hauser (1965), Reinauser et al. (1968) observed a decrease of pyruvate dehydrogenase (PDH) activity. Only in the case of thiamine deficiency developed by use of pyrithiamine, a marked reduction in pyruvate dehydrogenase was observed by Gubler (1961), Bennet et al. (1966) and Holowach et al. (1968). Considering that the activity of pyruvate dehydrogenase enzyme is reduced to some extent then the question arises about the presence of interconvertible active and inactive forms of this enzyme (Linn et al., 1969; Wieland and Jagow-Westermann, 1969). Secondly, as rightly pointed out by Dreyfus (1976b), "when one compares the loss of enzyme activity in the encephalopathy of thiamine deficiency (40-60 %) to that produced by a number of genetically determined metabolic encephalopathies known to be caused by an enzymatic defect, one realizes that the reduction in enzymatic activity in the tissues or blood of the symptomatic homozygote patient afflicted with

such a disease is usually very pronounced, sometimes in excess of 80 % while the asymptomatic heterozygote may show as much as 50% reduction in enzymatic activity. This fact alone casts serious doubts on the importance of enzymatic failure as the major cause of impaired neurological function in the encephalopathy of thiamine deficiency". This reasoning would not only apply to PDH but also to various other enzyme systems so far investigated.

In addition to the possible changes in the concentration of brain acetyl-CoA of the deficient rats, the following two plausible mechanisms have been suggested by Heinrich et al. (1973) :

1. a change in the catalytic activities or concentrations of ChAc and AChE ;

2. a change in the release of ACh from the presynaptic nerve endings.

With a view to study the first possibility we have carried out the investigation into the changes in the activities of brain AChE and ChAc in the deficient rats. The results of the same are presented in the following pages.

Effects of postweaning thiamine deficiency on brain enzymes :

The results of the studies on the effects of the thiamine deficiency on the brain enzymes are presented in Table 40. The average body weights were similar to those described earlier. Similarly, the profiles of the brain weight in each group were essentially same as in the previous experiment. It is interesting to note that protein levels were 11.82 ± 0.07 g % in the ad lib. control; 12.23 ± 0.24 g % in the pair-fed control; and 12.49 ± 0.13 g % in the deficient group. Inspite of the fact that the brain weights were significantly lowered as compared to ad lib controls, the protein levels of the deficient animals were significantly higher than those of the ad lib. group, ($p < 0.01$). But as compared to pair-fed group the protein levels remained unaffected. However, though DNA levels were not found to be significantly higher in the pair-fed (1.862 ± 0.015) and the deficient group (1.870 ± 0.011 mg/g) as compared to ad lib. controls (1.837 ± 0.005 mg/g), the difference between the pair-fed and the deficient animals was also not significant. The values for protein/DNA ratio were also interesting in a sense that this ratio essentially followed the pattern observed in the previous two parameters.

The elevation in the protein concentration was somewhat similar to that observed by Dreyfus (1976a).

Table- 40 : Effects of post-weaning thiamine deficiency on the brain constituents[1].

No.	G r o u p	Body wt. (g)	Brain wt. (g)	Protein (g.%)	DNA (mg/g)	Protein / DNA
1.	100 % B_1 ad lib. (8)[2]	133.0 ± 3.0	1.498 ±0.021	11.82 ±0.07	1.837 ±0.013	64.36 ±0.30
2.	100 % B_1 Pair-fed (8)[2]	65.0[a] ± 1.0	1.313 ±0.026	12.23 ±0.24	1.862 ±0.042	65.78 ±0.98
3.	0 % ad lib. (6)[2]	51.0[a,b] ± 1.7	1.241[a] ±0.024	12.49[a] ±0.13	1.870 ±0.022	66.85 ±0.99

Values are expressed as mean ± S.E.M.

1. The rats were sacrificed at the end of 5th or 6th week on establishment of frank neurological symptoms in thiamine deficient animals.
2. Numbers in parenthesis indicate number of rats in the group.

a = Values significantly different from ad lib. control ($p < 0.01$).

b = Values significantly different from pair-fed control ($p < 0.01$).

However, he reported significant increase in the brain levels of protein and DNA of the pair-fed and the deficient groups compared to the ad lib. group, but the difference between the first two groups was statistically non-significant. Dreyfus (1976a) reported decrease in the protein/DNA ratio between the pair-fed and the deficient group. Whereas, we could not observe similar difference; instead the ratio was increased as compared to the ad lib. group only. In view of the fact that the moisture content was not found to be affected, the possibility of that being artifact can be ruled out. However, in view of the fact that the protein/DNA ratio in the deficient group was not significantly different from that of pair-fed controls, these differences would not seem to contribute much to the gross differences in the brain cells.

As shown in Table 41, the activities of AChE and ChAc per gram brain weight remained unaffected in the deficient group. The activities of both the enzymes in the whole brain would be lower in the pair-fed as well as the deficient group. These deficits are , perhaps, due to the reduction in the brain size. In view of the observed increase in the protein concentrations one would expect the enzyme activities expressed on the basis of these two parameters to be lowered. Since, such a change

Table-41 : Effects of post-weaning thiamine deficiency on brain AChE and ChAc activities.[1]

	100 % B_1 ad lib.	100 % B_1 Pair-fed	0 % B_1 ad lib.
AChE			
(μmole substrate hydrolysed/min.)	(8)[2]	(8)[2]	(6)[2]
g brain	8.36 ± 0.38	8.85 ± 0.18	8.99 ± 0.27
mg Protein[3]	0.073 ± 0.003	0.072 ± 0.002	0.071 ± 0.16
mg DNA	4.71 ±0.20	4.70 ±0.12	4.81 ±0.14
ChAc			
(μmole ^{14}C-acetyl Ch formed/hr.)			
g brain	4.48 ±0.07	4.55 ±0.13	4.38 ±0.16
mg Protein[3]	0.038 ±0.001	0.038 ±0.001	0.035 ±0.001
mg DNA	2.44 ±0.021	2.44 ±0.04	2.38 ±0.10

Values are expressed as Mean ± S.E.M.

1. The rats were sacrificed at the end of the 5th or 6th week on establishment of frank neurological symptoms.
2. Numbers in parentheses indicate number of rats in the group.
3. Activity per mg of brain protein.

was not observed these differences, as mentioned earlier, do not seem to contribute much to gross changes in the brain and perhaps, are not of any functional value.

Heinrich et al. (1973) could not find any effect of thiamine deficiency on the activities of AChE and ChAc of the rat brain. As mentioned earlier, these workers also found reduced brain ACh and acetyl-CoA levels in the thiamine deficient rats. Since the activities of the enzymes involved in synthesis and break-down of ACh, were not affected the question would naturally arise as to how this change was brought about?.

It is pertinent to re-examine the "hypothesis" of reduced availability of acetyl-CoA for ACh synthesis in the brain of thiamine deficient rats. Wurtman and Fernstrom (1976) suggested that "susceptibility of neurotransmitter synthesis to the availability of precursors probably reflects an interplay of three biochemical mechanisms :

a) the propensity of rate-limiting biosynthetic enzymes (such as ChAc in the present case) to require, for full saturation, higher concentrations of their substrates than are normally present in the brain (Kaufman, 1974; White and Wu, 1973);

b) the inability of the brain to synthesize or store large amounts of these substrates and its consequent dependence on the circulation for obtaining them (Wurtman and Fernstrom, 1975; Ansell and Spanner, 1971) ; and

c) the tendency for substrate levels in plasma to vary within a considerable dynamic range, responding primarily, but not exclusively, to food consumption (Wurtman et al., 1968; Fernstrom and Wurtman, 1971; and Cohen and Wurtman, 1976) ".

Although the coupling of synthesis rates to precursor availability is a fairly widespread phenomenon in mammalian cells, the question of such a phenomenon occuring in the case of ACh synthesis, particularly in thiamine deficiency, should be most closely looked into.

As mentioned earlier, the brain ChAc appears to be unsaturated with its substrates in vivo, rat brain ChAc has a Km of 400 uM for choline and 18 uM for acetyl-CoA (White and Wu, 1973). The concentrations of choline and acetyl-CoA in the rat brain have been reported to be 37 uM (Cohen and Wurtman, 1975; Stavinoha and Weintrauh, 1974; Schmidt and Speth, 1975) and 7 to 11 uM (Sollenberg, 1970; and Shea and Aprison, 1975) respectively. In other words, both the

substrates seem to be much below the levels required for saturation of the enzyme of the two substrates. Choline availability for ACh synthesis has been very well studied. Cohen and Wurtman (1975) reported significant elevation in brain ACh and choline levels following the injection of increased dose of choline. In the further studies, it was also confirmed that increased dietary levels of choline resulted in elevated concentrations of ACh and choline in the different brain regions (Cohen and Wurtman, 1976; Growdon and Wurtman, 1979). Moreover, choline deficiency was found to lower brain ACh levels (Nagler et al., 1968). Similar correlation was also observed between elevated levels of the precursor amino acids in circulation (due to higher injestion of the same) and subsequent higher levels of biogenic amines in the brain (Wurtman and Fernstrom, 1976; and Fernstrom, 1977). Thus, it seems quite possible that elevation of substrate availability (due to manipulation of dietary levels or otherwise) definitely leads to subsequent greater synthesis of a neurotransmitter.

The other two biochemical mechanisms mentioned by Wurtman and Fernstrom (1976) would possibly, not apply to availability of acetyl-CoA as its brain level is not directly dependent upon the plasma level. Secondly, unlike choline, acetyl-CoA synthesis is not entirely dependent upon any exogenous source.

In view of the fact that ChAc is undersaturated with both the substrates, i.e., choline and acetyl-CoA, and the elevation of circulatory choline level results into increased concentration of brain ACh it would seem to be logical to presume the same correlation to underline between the availability of acetyl-CoA, the second precursor for ACh, and increased synthesis of ACh in the brain. Jope et al.(1978) studied the effects of various conditions which reduce the availability of acetyl-CoA in order to test the susceptibility of ACh synthesis in synaptosomes. The omission of glucose and/or succinate from the incubation medium resulted in decreased synthesis of ACh. Interestingly, the addition of bromopyruvate, inhibitor of pyruvate dehydrogenase, also resulted in decreased synthesis of ACh in the synaptosomes. There was a linear correlation between inhibition of pyruvate utilization due to bromopyruvate and the inhibition of ACh synthesis. However, the reduction in ChAc activity in the presence of bromopyruvate could not account for the observed inhibition of ACh synthesis. These authors are of the view that the cholinergic nerve terminals may contain a specialized type of pyruvate dehydrogenase for supplying acetyl-CoA for ACh synthesis. Secondly, their results also supported the suggestion of Blass and Gibson (1977) that reduced carbohydrate utilization may result in

decreased ACh synthesis prior to any changes in energy metabolism. Although, thiamine deficiency impairs the pyruvate utilization, it is not certain that this impairment could be compared with the bromopyruvate inhibition studies cited above. However, the conversion of (3-^{14}C) pyruvate into ^{14}C-ACh has been reported to be impaired in the brain homogenates in vitro (but not in vivo) following the thiamine deficiency and the treatment of the antagonists (Gubler, 1976a). Although these studies favour the contention that thiamine defieiency would decrease ACh synthesis through the impaired pyruvate utilization, the question of exact mechanism through which this change is brought about remains unresolved. One such possible mechanism could be the reduced availability of Acetyl-CoA which would not involve any change in the activities of brain AChE and ChAc.

However, to confirm this possibility much more detailed studies are warranted. In addition to this possibility, the non-coenzymatic function of thiamine in neural membrane requires detailed studies for possible correlation with cholinergic system in the nerve endings.

10. In vivo Studies on the Incorporation of the Radioactive lable into brain ACh from 1-^{14}C acetate and 3H (methyl) Choline :

From the foregoing discussion it is apparent that the deficiencies of protein, calories and thiamine seem to affect brain ACh levels in the rats. However, changes in ACh levels do not correlate well with the observed changes in the activities of the cholinergic enzymes. In view of this, it was felt worthwhile to study the effects on the incorporation of labelled precursors into brain ACh.

Similar isotopic techniques have been largely used in vivo as well as in vitro to estimate changes in ACh synthesis or to study the characteristics of the neuronal compartmentation of ACh in the central nervous system. Labelled ACh appears rapidly in the brain after intravenous, intracisternal, subcutaneous or tissue injections of either labelled choline or radioactive precursors of the acetyl moiety (Schuberth et al., 1969; Chakrin and Whittaker, 1969; Lefresne et al., 1973; Tuček and Cheng, 1974). As discussed earlier, various drugs have been shown to affect the rate of ACh synthesis in the brain. Turnover rate of ACh has been reported to be affected

by various drugs; these observations have been extended to different regions of the brain. However, there are limited number of studies reported with regards to nutritional deficiencies vis-a-vis rates of ACh synthesis using radioactive precursors.

In the present study, a very preliminary attempt has been made to throw some light on these aspects of ACh synthesis. The weaning rats were fed 5 % protein diet, thiamine deficient diet and the controls were pair-fed to the deficient animals. Since, the rats fed 5 % protein diet had comparable food intake only one control group was used. At the end of 6th week, 40 µCi each of 1-^{14}C acetate and 3H-(methyl) choline were administered intravenously; the rats were killed at 30,60 and 90 seconds, thereafter the brain samples were processed, as detailed earlier, to get renickates of ACh and choline, separated by chromatography, and counted in the liquid scintillation counter using appropriate window settings and making corrections for quenching and "spill over".

The results of the same are presented in Figures 10 and 11. As can be seen from the figures, the number of observations was very much limited. Hence it would not be possible to evaluate the results quantitatively. These can, at the best, serve as mere pointers. It is admitted that

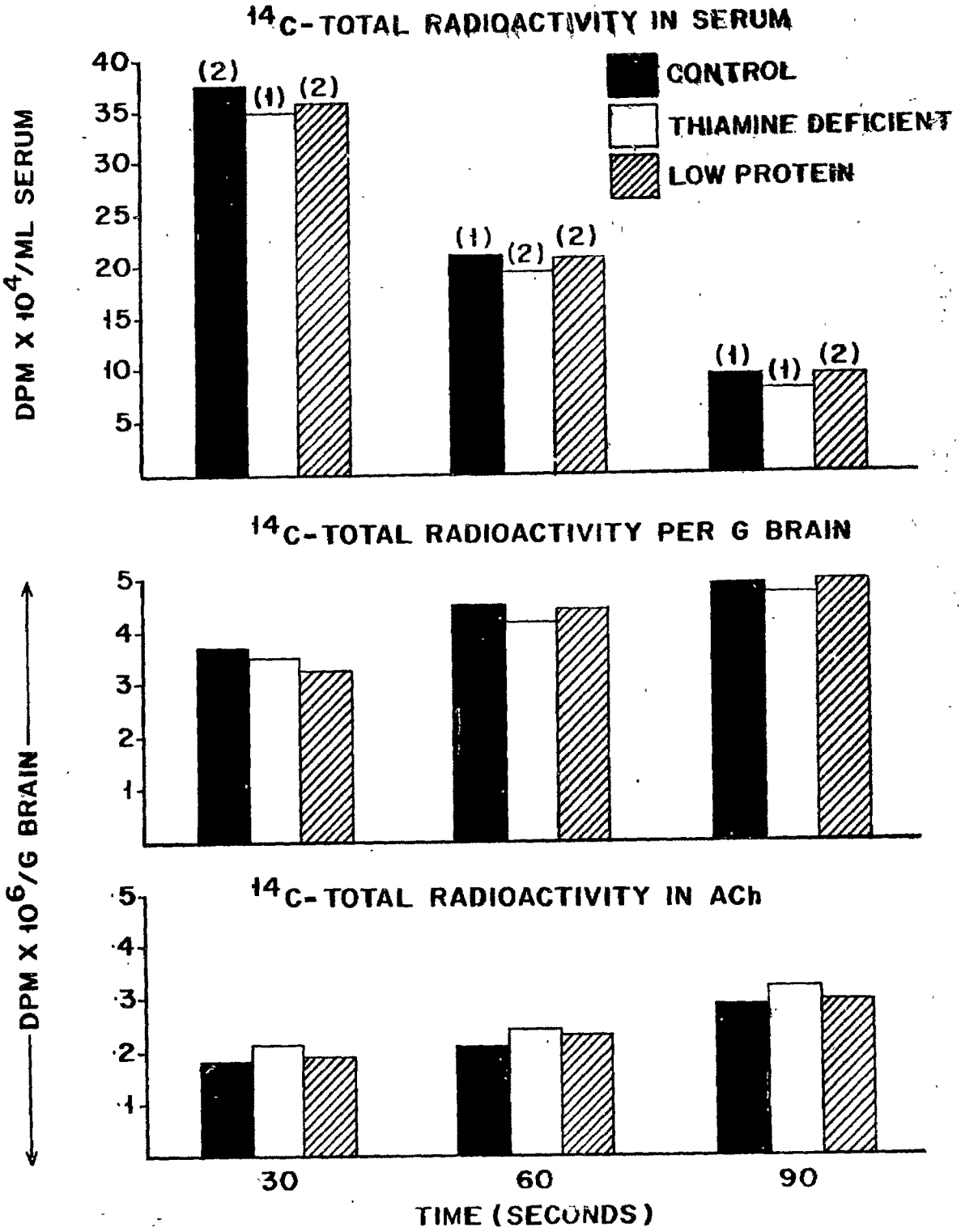

Fig. 10- Rats were killed 30,60 and 90 seconds after administration of 40 uCi each of ^{14}C-acetate and 3 H (methyl) Choline. Values in brackets indicate number of observations.

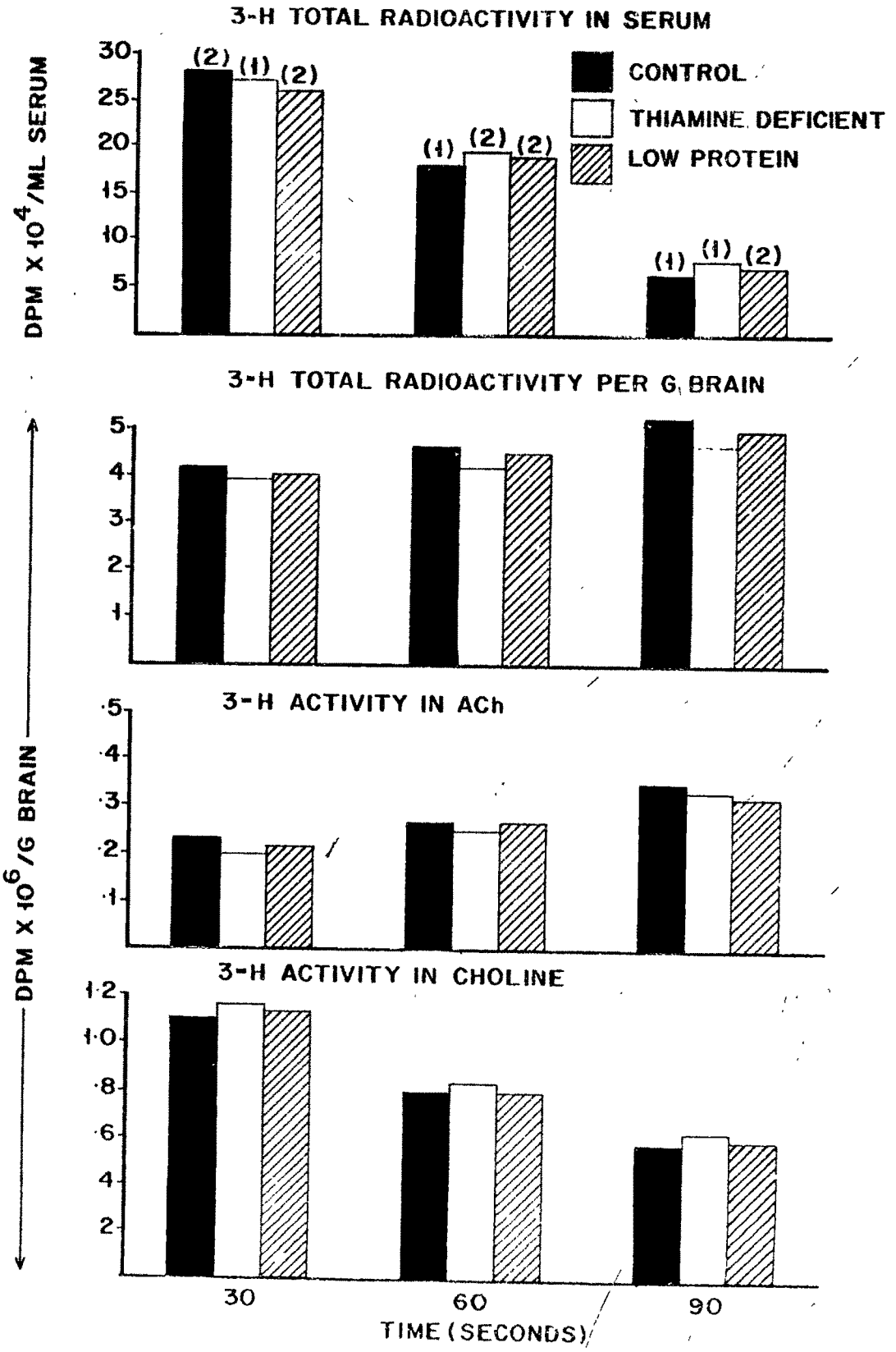

Fig 11- Rats were killed 30,60 and 90 seconds after administration of 40 μCi each of ^{14}C acetate and 3 H(methyl) Choline values in brackets indicate number of observations

more detailed studies are required to be carried out to augment these observations.

As can be seen from Figure-10, ^{14}C activity in serum decreased with the progress of time after injection of the precursors. Apparently there is no difference in any of the three groups of animals; ^{14}C total activity in the brain increased with the progress of time. About 4% of the injected ^{14}C activity was retained at the end of 30 seconds, eventually progressing to 5.5 % at the end of 90 seconds. ^{14}C Activity in ACh per g brain increased from 0.18 DPM X 10^6 at 30 seconds to 0.32 DPM X 10^6 at the end of 90 seconds. However, there is, apparently, no difference in the three groups. The radioactivity in ACh per g brain at 90 seconds constitutes 0.30 % of the injected and 6 % of the total radioactivity in the brain.

3H Radioactivity at different times after injection of the precursors is presented in Figure-11. The radioactivity in the serum decreases with the time. However, the activity is lower as compared to ^{14}C radioactivity in the serum. Apparently, there is no difference in the profiles of the three groups. 3H Radioactivity in the brain was found to increase with the time. However, it constitutes 0.4 % of the injected activity and 7 % of the total radioactivity in the brain. 3H Radioactivity in

choline per g brain decreased with the time. The ratio of radioactivity of acetylcholine to choline in the control was 0.21 at 30 seconds and 0.55 at 90 seconds. This suggests the linear incorporation of 3H-choline into brain ACh.

As mentioned above, the quantitative evaluation of the data is not possible in view of limited observation. However, apparently there seems to be no significant difference in the incorporations rates of the label into ACh in the brain of thiamine and protein deficient rats. The ^{14}C radioactivity in brain ACh of the control group as percent of injected radioactivity was less compared to 3H radioactivity in the same. This may be due to the fact that incorporation of ^{14}C from 1-^{14}C acetate into brain lipids is several times more as compared to other substrates (Tuček and Cheng, 1974). Secondly, pyruvate and glucose seems to be more important precursor of the acetyl group of ACh (Sollenberg and Sorbo, 1970; Tuček and Cheng, 1974).

It is interesting to note in this connection that the incorporation of ^{14}C label into brain ACh from (3-^{14}C)-pyruvate <u>in vivo</u> was not found to be affected in thiamine deprived and oxythiamine treated rats, whereas, the pyrithiamine administration resulted in higher incorporation

into brain ACh (Cheney et al., 1969). Interestingly, in vitro studies revealed decreased incorporation of the label from (3-^{14}C) pyruvate in both oxythiamine and pyrithiamine rats but not in the thiamine deprived rats (Cheney et al., 1969).

In summary, it can be said that present study needs to be augmented with much more number of observations and extension to investigate other aspects like incorporation rates of various precursors, estimation of indegenous choline levels, etc. Further studies are warranted to throw more light on the synthesis of brain ACh, in particular, its turn-over rate in the deficient animals.