CHAPTER 4

EFFECT OF AMMONIA LOADING ON GLYCOGEN CONTENT AND PHOSPHORYLASE AND GLYCOGEN SYNTHASE ENZYME ACTIVITIES IN THE GLYCOGEN BODY OF 5 DAY OLD POST-HATCHED CHICKS OF DOMESTIC FOWL, *GALLUS DOMESTICUS*.

It is well known that ammonia, at elevated concentration is toxic to the central nervous system (C.N.S.) and this increased concentration is the potential complicating factor in the neurological abnormalities produced by several diseases (Cooper and Plum, 1987). Ammonia is readily permeable into cells due to its uncharged nature and high lipid solubility. On entry it accepts protons (H^+) which results in a rise in pH away from optimum (Roos and Baron, 1981; Erecinska *et al.*, 1987). In experimental animals ammonia is known to cause seizure and coma (Navazio *et al.*, 1961; Tsukada, 1971; Hindfelt, 1975 ; Ehrlich *et al.*, 1980). Astrocytes are the brain's glia that are known to act as " enzymatic barrier" to ammonia that is derived from both the blood and brain (Cooper and Plum, 1987). Ammonia is known to cause morphological abnormalities in astrocytes *in vivo* (Cole *et al.*, 1972; Gutierrez and Norenberg, 1977; Voorhies *et al.*, 1983) and in primary astrocyte cultures (Gregorios *et al.*, 1985). Severe liver diseases usually lead to

hyperammonemia, which in turn is thought to play an important role in encephalopathy wherein accumulation of glycogen in the astrocytes take place (Sherlock *et al.*, 1954; Walker and Schenker, 1970; Gerok and Haussinger, 1984). In spite of considerable work that has been done to reveal the effects of ammonia on brain function, the exact mechanism underlying ammonia toxicity is still obscure. Most of the studies pertaining to ammonia toxicity have been carried out either in cultures or in mammalian systems *in vivo*. Such work in birds and especially on astrocytes is truly lacking.

Astrocytes of birds form an easily removable mass of tissue. Glycogen body is a gelatinous mass of specialized astroglial cells that fills the lumbosacral sinus of the avian nerve cord at about 7-8 days of incubation (De Gennaro, 1982) and hence serve as a good working material for carrying out quantitative studies on ammonia related toxicity. Moreover the subject matter has bearing on oft made queries by the workers working on glycogen body *viz.* 1) why has this strucutre arisen in birds ? 2) why should it appear in lumbosacral area ? 3) why should the cells proliferate on days 7,8 ,9 and 10 ? 4) why should they accumulate glycogen? Although it is beyond the capacity of a single worker to find answers to all these

queries within a span of few years, at least there is scope for extrapolation if one knew the effects ammonia had on different components of C .N. S., at least at a specific dosage, for , during avian development ammonia is known to accumulate in allantoic sac on day 7th, the day on which glycogen body cells begin to proliferate. Moreover, fairly large quantities of glycogen begin to accumulate in glycogen body cells around this time of development (Watterson, 1949). The physiologic function of this glycogen reserve yet remains to be firmly established (Chapter 1). Researchers from this laboratory (Nene and Syeda, 1988; Syeda and Nene, 1990) have surmised that the glycogen body may be functioning as a detoxifying tissue conferring protection to the C.N.S. similar to that conferred by the liver to the whole body. Hence an attempt has been made to study the effect of sublethal dose of ammonium acetate on glycogen content and its related enzymes *viz.* glycogen synthase and glycogen phosphorylase in the glycogen body of 5 day old neonatal chicks of domestic fowl, *Gallus domesticus* with a view to understand its functional significance in these animals. Liver was also processed for the estimation of glycogen for comparison.

MATERIAL AND METHODS

Day old chicks of domestic fowl, *Gallus domesticus* (Rhode island Red variety) were procured from government poultry farm. They were maintained in cages and offered water and fed chicken feed *ad libitum*. On day 5, the chicks were divided into three groups of 40 each and treated as follows :

Group I : Control birds [Received 0.5 ml of distilled water per kg of body weight].

Group II : Experimental birds [Received 0.4 ml of 1 mM (1/50th of LD_{50}) ammonium acetate in distilled water per kg of body weight].

Group III : Experimental birds [Received 0.4ml of 5 mM (1/10 th of LD_{50}) ammonium acetate in distilled water per kg of body weight].

Administration of LD_{50} dose to chicks resulted in contraction of extensors leading to stretching of legs, gradual closure of eyelids, defecation and also inability to balance itself. After an interval of 10 minutes, however, they regained postural normalcy. Hence the two

sublethal doses of ammonium acetate mentioned above were selected for the present work. The chicks in all the three groups received injections intraperitoneally. Fifteen minutes following injection, they were sacrificed by decapitation and later exsanguinated. Glycogen body tissue was quickly dissected out, blotted free of body fluids and weighed on an electronic balance to be processed later for the following biochemical analysis.

Glycogen : Glycogen in the glycogen body and liver were assayed using the Anthrone method of Seifter *et al.* (1950) and the glycogen content expressed as mg/100 mg wet tissue.

Protein : Protein content of glycogen body was estimated according to the method of Lowry *et al.* (1951) using Folin-Phenol reagent with Bovine Serum albumin as standard and expressed as mg/100 mg wet tissue.

Glycogen synthase(EC 2.4.1.11, UDPG α-glucan glucosyl transferase) : The activity of enzyme glycogen synthase in the glycogen body was determined using the method of Leloir and Goldemberg (1962).

Phosphorylase(EC 2.4.1.1, 1,4-α-D-glucan :Orthophosphate,

α-D-glucosyl transferase) : The phosphorylase enzyme activity in the glycogen body was assayed according to the method of Cahill *et al.* (1957).

Statistical difference between the experimental and control samples were done using the student's 't' test.

RESULTS

The protein concentration of glycogen body in both the control and ammonia treated chicks were almost constant and did not show any significant difference (Table II, Fig. 4). Similarly the activity of enzyme glycogen synthase also did not show any significant alteration (Table II, Fig. 3) when the experimental data were compared with that of the controls. However, the glycogen content of the glycogen body and its phosphorylase enzyme activity were drastically affected in both 1mM and 5mM ammonium acetate treatment; whereas glycogen load increased significantly (Table I, Fig. 1a). phosphorylase activity showed a significant decrease (Table II, Fig. 2). The liver, on the other hand. following ammonia treatment, showed a significant increase in glycogen content only in 5mM treated material; 1mM dose failed to generate a significant response in this tissue (Table I, Fig. 1b).

Table I : Effect of ammonium acetate on glycogen content of glycogen body and liver in mg % tissue wet weight.

TISSUE	CONTROL	AMMONIUM ACETATE	
		1mM/kg b.w.	5mM/kg b.w.
Glycogen body	28.85 ±0.738 (10)	40.03^{c} ±1.677 (10)	47.54^{c} ±2.347 (10)
Liver	1.89 ±0.384 (07)	2.37 ±0.309 (07)	4.90^{c} ±0.431 (07)

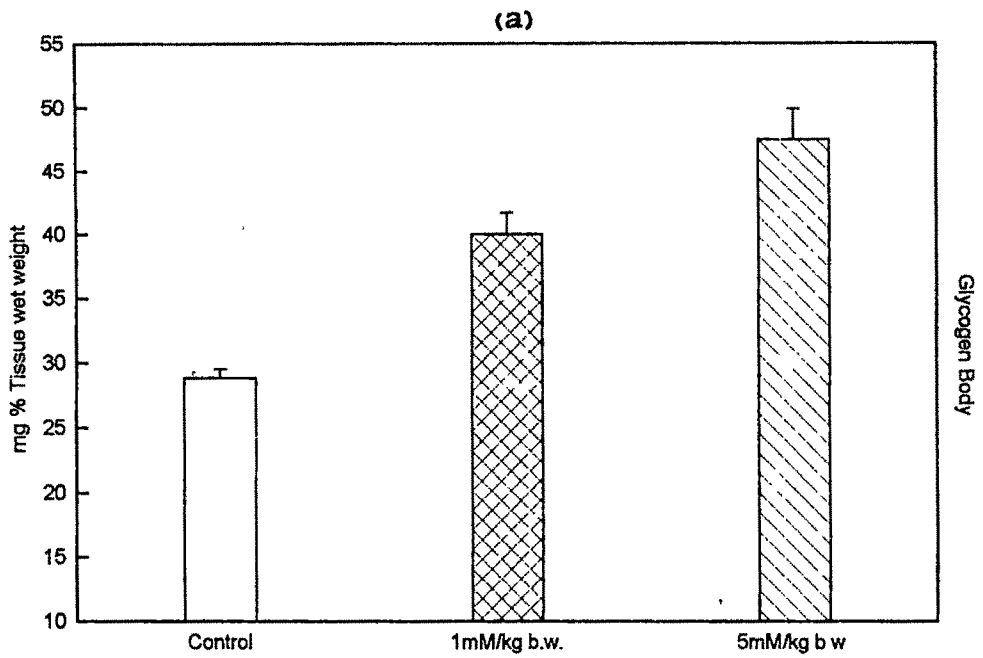
Values are mean ± SEM. Figures in parentheses indicate the number of assays. Significantly different from the values for control by 't' test are indicated as $^{c}p< 0.001$.

Table II : Effect of ammonium acetate on total protein, glycogen synthase and phosphorylase enzyme activites of the glycogen body of 5 day old neonatal chicks of domestic fowl, *Gallus domesticus*.

PARAMETERS	CONTROL	EXPERIMENTAL	
		1mM/kg. b.w	5mM/kg. b.w
Protein(mg/100mg wet weight)	4.73 ±0.23	5.08 ±0.69	5.17 ±0.26
Glycogen synthase(uM UDP formed/mg pro/10 minutes)	0.047 ±0.008	0.058 ±0.009	0.067 ±0.002
Phosphorylase(ug P released/mg pro/10 minutes)	343.54 ±14.35	198.95^{c} ±11.71	49.65^{c} ±4.54

Values are mean ± SEM of 6-10 assays. Significantly different from the values for control by 't' test are indicated as $^{c}p< 0.001$.

Figure I Effect of ammonium acetate on glycogen content of glycogen body and liver.



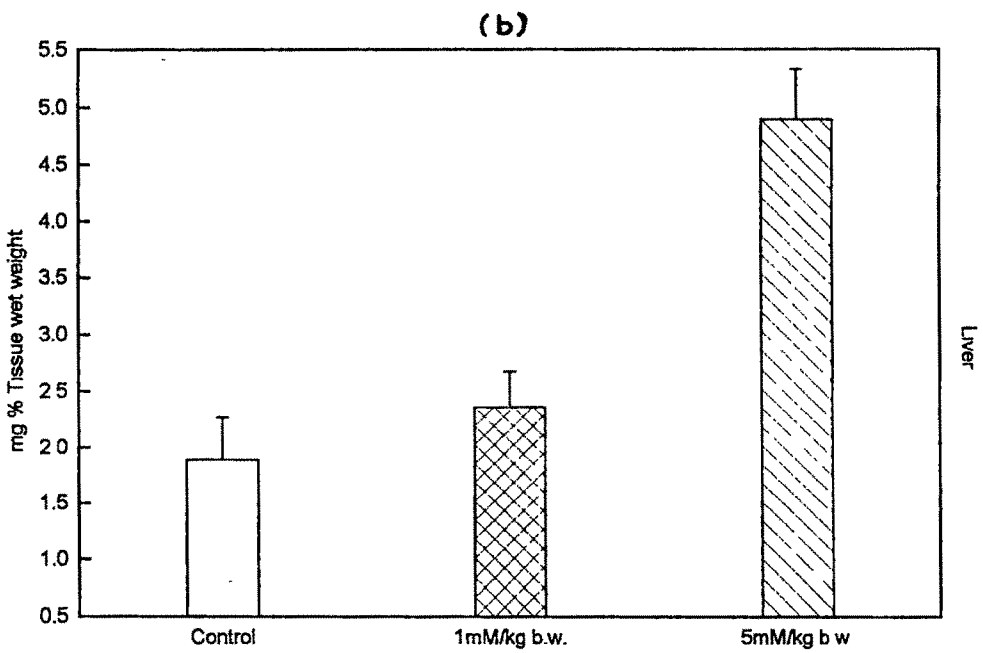


Figure 2. Effect of ammonium acetate on phosphorylase activity of the glycogen body of 5 day old chicks of domestic fowl, *Gallus domesticus*

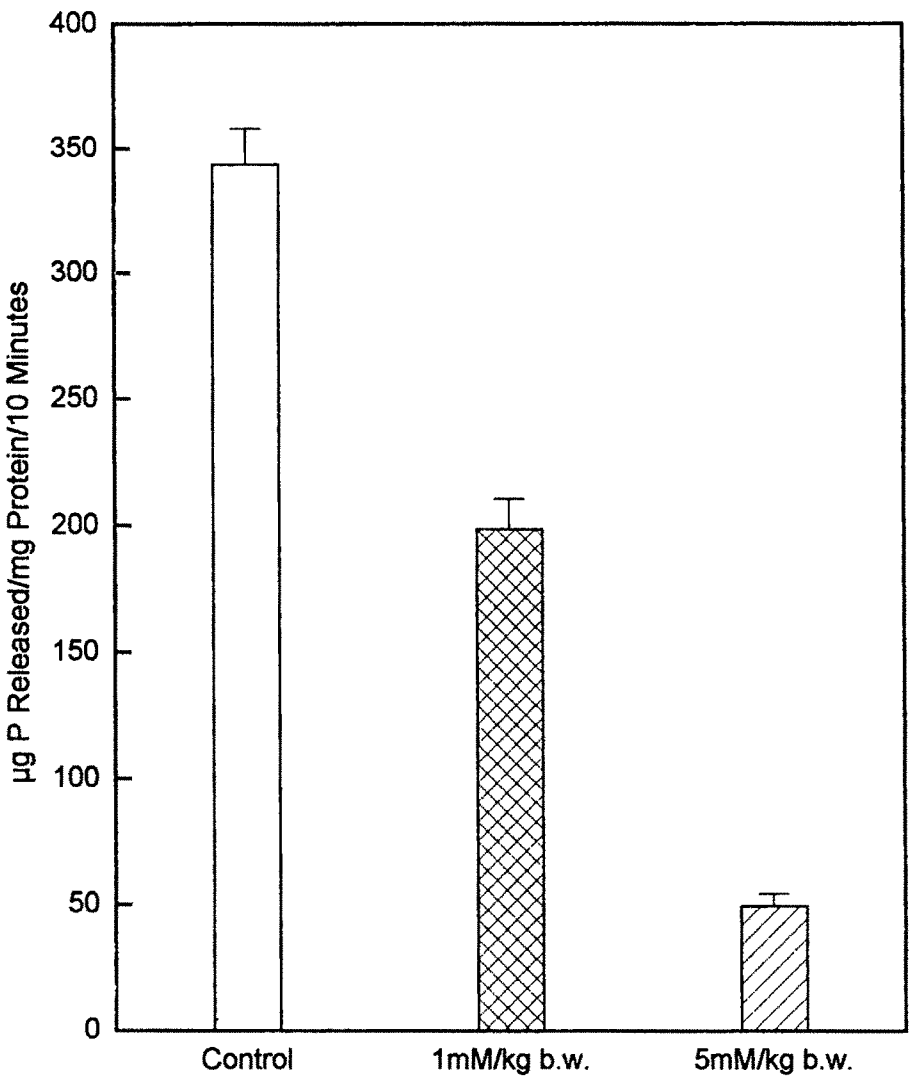


Figure 3. Effect of ammonium acetate on glycogen synthase activity of the glycogen body of 5 day old chicks of domestic fowl, *Gallus domesticus*

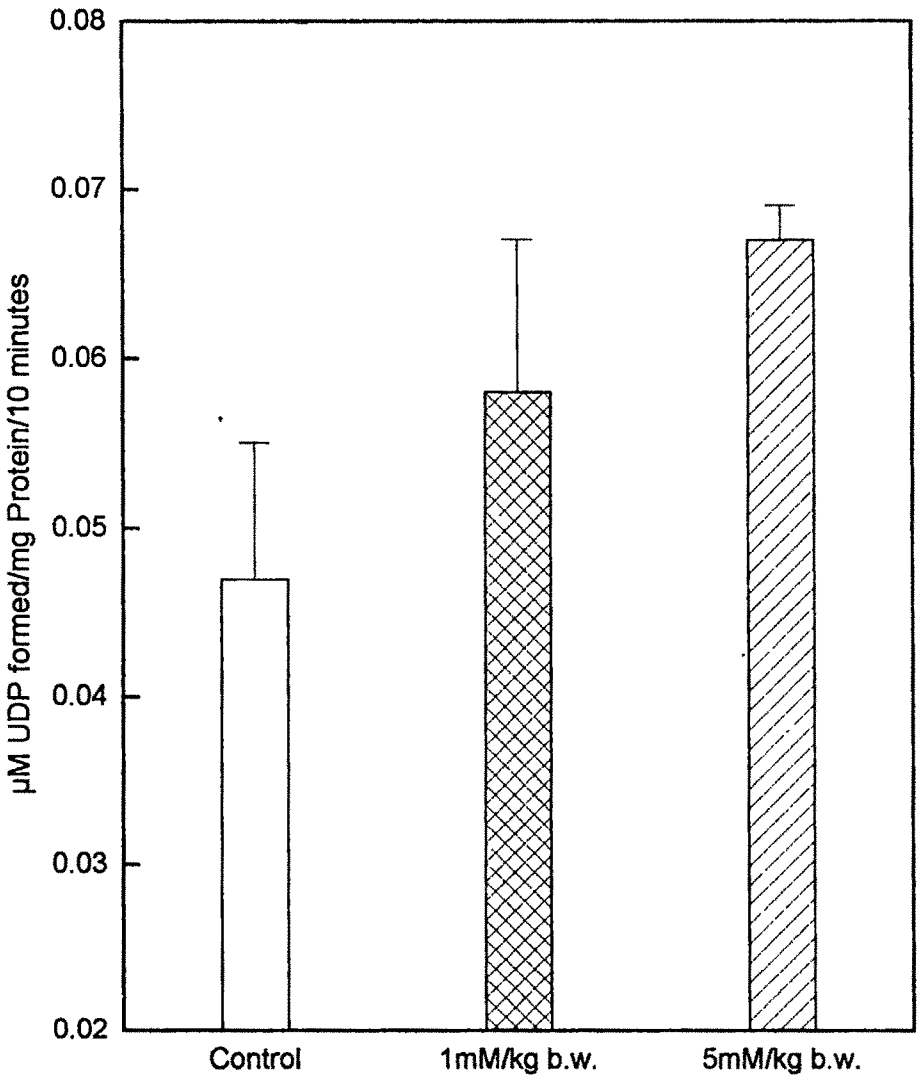
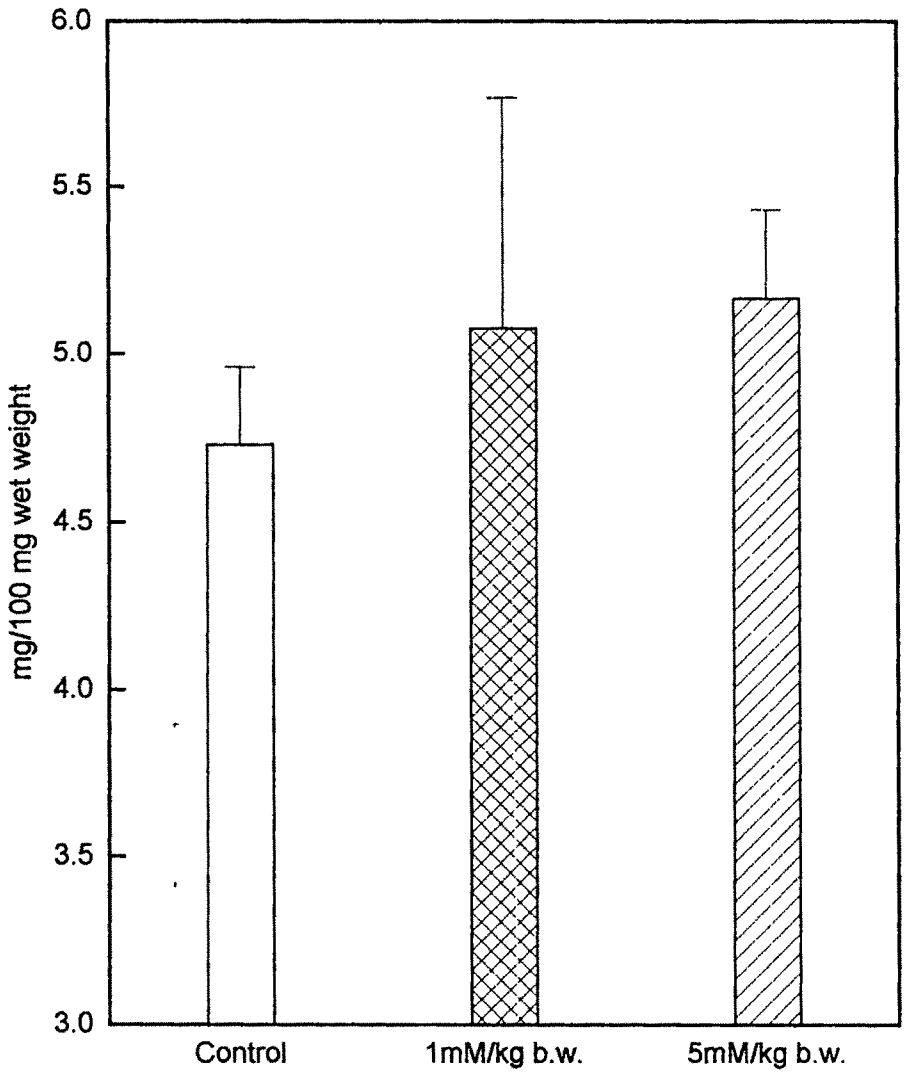


Figure 4. Effect of ammonium acetate on protein content of the glycogen body of 5 day old chicks of domestic fowl, *Gallus domesticus*



DISCUSSION

Hepatic encephalopathy, a disease characterized by increased concentration of ammonia in the brain is quite well known (Walker and Schenkar, 1970). Ammonia enters the brain from blood by diffusion. Ammonia free base (NH_3) has a pK of 9.1. At normal blood pH of 7.4 (mammals), approximately 98% is in ionized state (NH_4^+) and hence not diffusible. However, at higher pH a greater proportion is in the gaseous state(Cooper and Plum, 1987), hence, due to its high lipid solubility would permeate cell membranes more freely than does ammonium ion (Lawerence *et al.*, 1957; Warren and Nathen, 1958). Astrocyte is known to be the critical compartment where ammonia is detoxified. Increased glycogen in brain has been demonstrated following use of anesthesia (Brunner *et al.*, 1971), X-irradiation (Nelson *et al.*, 1968; Lajtha, 1971) and neuronal injury (Ibrahim *et al.*, 1972). Astrocytes undergo several pathological changes in severe, acute and chronic liver disease and hence play some essential role in carbohydrate metabolism of brain.

Glycogen body, an accumulation of specialized astroglial cells is known to store glycogen in its cytoplasm during embryonic development of avian nerve cord (De Gennaro,

1982). An approach towards understanding the role of this tissue in carbohydrate metabolism provided an impetus to work on ammonia induced effects in the chicks. Intraperitoneal injection of ammonium acetate (both 1mM and 5mM per kg of body weight) resulted in remarkable increase in glycogen content of glycogen body. Liver, however, reacted differently. Although at the dose of 5mM per kg of body weight there was an increase in glycogen, administration of 1mM per kg of body weight of ammonium acetate failed to show significant increase, indicative of ammonia detoxification. Adult avian liver has the ability to detoxify exogenous ammonia (Wilson *et al.*, 1968 and Karasawa *et al.*, 1980).

Following intraperitoneal injection of ammonium acetate. NH_3 is absorbed via the portal circulation system to pass through the liver before entering the systemic circulation. As the capabilities of the detoxification enzyme system are surpassed the excess ammonia enters brain from blood through a disturbed blood-brain barrier thus exerting its potential toxic effects. Liver shows a high glutamate dehydrogenase activity in chicks, in contrast to glycogen body which possesses only moderate quantities of the same (Syeda, 1989).

Liskowsky *et al.*(1986) have postulated that ammonia disrupts normal astrocyte function by bringing about perturbation in the adrenergic cAMP producing system leading to dephosphorylation of proteins and inactivation of enzymes especially phosphorylase which is pH sensitive. A significant decrease in the activity of enzyme phosphorylase was observed following ammonium administration. Hence the observed increase in glycogen may be due to inhibition of phosphorylase brought about as a result of ammonium ion accumulation within the cells thereby rendering the intracellular milieu alkaline and therefore not optimal for the activity of enzyme phosphorylase. Danforth (1965) had also found phosphorylase to be highly pH sensitive, a change towards alkalinity rendering it inactive. Similarly Sahu and Bose (1976) have reported the phosphorylase in glycogen body of chick to become inactive under alkaline pH. Another possible reason for inhibition of phosphorylase could be as follows. It is known that this enzyme requires pyridoxal phosphate as a cofactor and normally hydroxyl moiety of pyridoxal phosphate forms a Schiff base with Ɛ-amino group of lysine of the enzyme. It is possible that entry of ammonia into the cell changes this picture and pyridoxal phosphate now combines with an ammonium derivative so as to form Schiff base depriving the enzyme

the opportunity to interact with pyridoxal phosphate and hence shows a decrease in its activity.

Subacute dose of ammonium acetate did not show any marked changes in the activity of enzyme glycogen synthase in the glycogen body of chicks. Increases in glycogen with no significant increase in glycogen synthase could be explained on the basis of following information. Hexokinase activity is seen to increase with increasing pH. Thus G-6-P formed could be converted to G-1-P which can subsequently react with UTP to form UDPG. Also ammonia was found not to affect uptake of glucose in rat astrocytes (Dombro *et al.*, 1993).

Sublethal dose of ammonium acetate also did not show any marked change in protein concentration of glycogen body. Inhibition of protein synthesis in brain of rat following ammonia challenge has been observed by some (Brun *et al.*, 1977; Hindfelt *et al.*, 1979: Schott *et al.*, 1985: Subrahmanyam *et al.*, 1985). Contrary to this, Minana *et al.* (1988) have shown increased brain protein synthesis. Brun *et al.* (1977) are of the opinion that the protein loss may be largely neuronal and may be secondary to abnormalities of glial function.

Work on brain cell energetics in rats show no significant change after treatment with acute dose of ammonium acetate

(Hindfelt and Siesjo, 1970, 1971; Hawkins *et al.*, 1973), nor does any change occur in the level of C-ketoglutarate (Hawkins *et al.*, 1973; Mans *et al.*, 1979). Even the levels of glutamate have been shown to remain unchanged following ammonia intoxication (Berl *et al.*, 1962; Clark and Eiseman, 1958; Bradford and Ward, 1976). There are reports which say that in hyperammonemic rats, the increase in glutamine content in the brain produces an osmotic shift in water resulting in cerebral edema. Pilbeam *et al.* (1983) have suggested that high circulating levels of ammonia may produce ionic shifts that results in interference with nervous function *in vivo*. Ammonium ion also produces increased cytoplasmic osmolarity as a result of which the astrocytes appear swollen in the electron micrographs and hence brings about derangement of astrocytes (Norenberg, 1981). Cole *et al.* (1972) and Norenberg *et al.* (1974) have shown increased astroglial proliferation in the brain of rats in response to liver disease. Electron microscopy of urease treated rats demonstrated astrocytes with enlarged cytoplasm and increased number of mitochondria. A prominent feature of the astrocytes in portocaval shunted rats (Zamora *et al.*, 1973; Norenberg, 1977) were an increased amounts of cytoplasmic glycogen in them. The morphological changes observed according to Zamora *et al.* (1973) represent an

increased metabolic activity directed at the detoxification of ammonia. The meaning of the increased glycogen in astroglial cells is uncertain. The accumulation may be due to decreased neuronal activity or a non-specific change, found in various experimental states and generally assumed to reflect injury to these cells.

It is of interest that during development of chick embryo, a similar situation occurs on day 7. It is on this day that ammonia appears in allantoic sac for the first time (Romanoff, 1967). Precisely during this time the astrocytic cells in the lumbosacral region divide and accumulate glycogen. This process continues for another 3 days until these cells form a wedge shaped structure called the glycogen body. Cells of this glycogen body tissue envelope the central canal at the lower end and at the same time project dorsally into the subarachnoid space. Concurrently there occurs an increase in its glycogen content. This is a situation analogous to the results reported earlier. On the basis of these results, therefore, one is tempted to speculate on the possible effect ammonia may have on the developing avian embryo unsupported as it is by a structure equivalent of mammalian placenta capable of speedy removal of ammonia produced by the embryo. During development of C.N.S.,

there occurs polyneuronal burst activity on the 3rd day of embryonic development in the cranial region of the spinal cord of chick, to be followed in the thoracic region on the 4th day of incubation and finally in the lumbosacral part on the 5th day (Porvine *et al.*,1970). At the lumbosacral spinal cord level, however, accumulation of toxic material such as ammonia, produced as a result of this burst and increased neuronal activity (Cooper and Plum, 1987) seems to have a wide ranging repercussions on the local population of cells, especially the subependymal glial cells. In order to protect subglial tier of neurons and their processes it is this tier of subependymal glial cells which have to bear the brunt and carry out the mopping up operation. In so doing their own intracellular environment changes, to become alkaline providing a signal for mitosis. A rapid increase in intracellular pH may be important in bringing cells from Go/G1 into S phase (Gerson and Burton, 1977; Gillies and Deamer, 1979; Pouyssegur *et al.*, 1985; Ober and Pardee, 1987; Madshus, 1988) and initiate the process of DNA synthesis. It is seen that prior to burst activity the spinal cord undergoes occlusion except in the region of lumbosacral spinal cord (Schoenwolf and Desmond, 1984). CSF remains trapped in the neural canal at the lumbosacral level. It seems plausible that the liver which is not yet fully mature, may not be in a position to remove the

excess ammonia generated as a result of polyneuronal burst activity and therefore accumulates in the lumbosacral region of the spinal cord probably bring about induction of mitosis as a result of local rise in pH. As explained earlier this would also put out phosphorylase activity leading to increase in the glycogen load of the cells in the roof plate of nerve cord. In chick embryo, spinal neurons are endogenously active and generate an output to muscles by embryonic day 3.5 (Oppenheim, 1991). However, large mass of ventromotor neurons die between embryonic day E6-E12 in the lumbar spinal cord of the chick (Oppenheim, 1991). Increased neuronal activity accompanied by death of neurons would generate ammonia (Cooper and Plum, 1987). Loss of ventrally located supporting structures in all probability causes caving in of the tissue located above, drawing glial cells immediately ventral to centrally located neural canal to more ventral position. Neural canal in the lumbosacral area, therefore, comes to occupy a more ventral location than elsewhere (see Fig.3, Introduction). As a result of this upheaval, the glial cells lying above the neural canal now occupy the bottom of the pit formed as a result of caving in of the roof of the nerve cord. These glial cells due to the reasons explained earlier now multiply, their daughter cells are pushed up and fill up this pit and continue to spread dorsally into the newly

created space. Their mass invasion dorsalward culminates into the formation of a wedge shaped structure, causing alteration in topography of lumbosacral region. At about this time, other cell types in the lumbosacral region continue uninterruptedly the work assigned to them according to the morphogenetic dictates. Even so cells that are destined to form pia mater, finding themselves at a location different (i.e. at the bottom of the pit) than what was planned for them, now have to move along the edge of the newly created wedge shaped mass of glycogen body. As a result, a topographically different structure gets formed. This is exemplified by transverse shelf, a unique structure found only in the LS, formed by pia mater at the periphery of glycogen body tissue. The analogy viewed against morphogenetic backdrop helps explain all the queries raised in the early part of this chapter. It has shown what far reaching effects imposition of a single factor can have in upsetting the developmental schedule. Till the time the nature of the peptides formed by glycogen body and the material contained in golgi is not known all one can say is that the unique wedge shaped structure found mid-dorsally, laden with huge quantities of glycogen, found only in lumbosacral region of aves appears to be a product of several forces acting on the nervous system of the embryo during days 3 to 12 in chicken. These forces being

generation of ammonia that cannot be halted, absence of a mechanism for its speedy disposal, its accumulation in LS, effort made by glial cells for its removal, in the process, glial cells upsetting their own developmental plan, cells now laden with the intracellularly heavy load of glycogen weighing on the thinning delicate hollow mass of tissue beneath, crumbling of the roof of the dilapidated structure below with consequent creation of a conical space which never existed before, the need to fill up the space and rounding up of this matter within its fold by the prompt, spreading and enveloping pia mater. The culmination of these various factors acting on the nervous system of embryo would be formation of a pearl-like mass.