

CHAPTER VII

Fluoride Induced Changes in the Glucose Transport and Uptake Mechanisms of Liver and Muscle of the Ex-ovo Developing Chick, *Gallus gallus domesticus*.

The autonomic nervous system (ANS), is a major modulator for regulating metabolic activities through its action in liver as well as in several endocrine glands. Through its two major components, sympathetic and parasympathetic, ANS features prominently in glucose regulation between meals, during periods of starvation, or in response to an environmental change or stress, not involving meal ingestion (Porte and Halter, 1981). Parasympathetic stimulation results in release of glucose from the liver into the circulation (Lautt, 1980). Moreover, acetylcholine (ACh), the cholinergic neurotransmitter is a direct stimulant of insulin secretion, while nor-epinephrine (NE), the adrenergic neurotransmitter, is a direct stimulant of glucagon release from the islets (see review by Shimazu, 1983).

The idea of participation of cholinergic system in the assimilation of metabolites has taken root quite early. Bertly (1954), observed cyclic variations in the intralobular localization of hepatic cholinesterase during feeding and fasting. Gerebtzoff (1959) indicated a relationship of diet with concentration and distribution pattern of cholinesterase in the rat liver. Being endowed with both cholinergic and adrenergic fibres, the avian liver too must be under their influence as far as assimilation is concerned. Pilo (1967), through his studies on hepatic cholinesterases in a migratory and some non-migratory birds, has suggested that acetylcholine-acetylcholinesterase system in some way or the other is involved in the metabolism of carbohydrates and lipids in the liver. Mondon and Burton (1971) clearly demonstrated that the acetylcholine or choline, in the presence of insulin significantly enhanced the uptake of glucose and deposition of glycogen by the liver of rats. It is possible then, that in the avian liver too, this cholinergic system is playing a prominent role in the uptake of glucose. Moreover, it has been recorded that cholinergic impairment leads to hyperglycaemia in pigeons (John *et al.*, 1985; Oommen, 1992).

Chronic fluoride intoxication caused hyperglycaemia in post-hatched developing chicks (Chapter 6). Fluoride is known to enter the brain and blood-brain barrier fails to exclude it

from the nervous tissue (Geeraerts *et al.*, 1986). The manifestations of the initial phase of fluorosis indicate injury to the central nervous system and the spinal cord (Waldbott *et al.*, 1978). Hence, it is possible that an altered sympathetic-parasympathetic equilibrium, set in due to fluoride induced neuropathy, could be one of the reasons for the elevated glucose level in fluoride poisoned chicks. Since the level of activity of acetylcholinesterase (AChE) could be taken as an index of acetylcholine secretion, AChE could be used as a yardstick to judge the degree of ACh secretion. Hence in the present study it was thought worthwhile to evaluate the activity of AChE in the liver and gastrocnemius muscle of fluoride treated chicks.

Furthermore, if ACh facilitates glucose transport, there is sufficient data to suggest that this must be taking place through flow coupled transport, which is one of the mechanisms by which cell accumulate glucose (Wilbrandt, 1975). The ACh could initiate permeability changes that bring about movements of ions across the plasma membrane of hepatocytes, probably through the release of membrane bound calcium ions. This alteration in the permeability results in an influx of Na^+ into the liver cells followed by outflux of K^+ into the extracellular fluid. Sodium pump is immediately activated and Na^+ extrusion and K^+ re-uptake processes start to function. During these processes glucose is taken into the hepatocytes utilizing the same energy and carrier molecules (see review by Pilo and Verma, 1985). Since Na^+-K^+ ATPase is the enzyme involved in the transport of ions across the plasma membrane and as cholinergic inhibition (vagotomy) was found to curtail the activity of this enzyme (Pilo *et al.*, 1984), the present study endeavours to estimate the activity of Na^+-K^+ ATPase in the liver and gastrocnemius muscle of fluoride intoxicated growing chicks to varify the present notion of fluoride induced parasympathetic neuropathy.

Another set of enzymes which are involved in the transport of metabolites across the cell membrane are non-specific acid and alkaline phosphatases. Acid phosphatase, according to the functional state of the tissue concerned, is reputed to be involved in a number of activi-

ties such as phagocytosis (Klockars and Wegelius, 1969), dissolution of tissue components (Weber and Niehus, 1961), fat absorption in intestine (Barka, 1963), cellular differentiation (Ghiretti, 1950) and Keratinization (Novikoff, 1961; Mishima, 1964; Palade and Forquihar, 1965). While alkaline phosphatase has been associated with a number of activities such as formation of fibrous protein (Verzar and Mc Dougall, 1936; Moog, 1946; Bradfield, 1950), calcification of bones (Moog, 1944; Pitchard, 1952) and phosphate transfer in DNA metabolism (Rogers, 1960). This enzyme is also reported to be involved in carbohydrate metabolism (Cori and Cori, 1952; Cusworth, 1958; Duncans, 1959; Rosenthal *et al.*, 1960).

Quantitative estimations of non-specific acid and alkaline phosphatases were therefore undertaken in the present study to understand the relationship of fluoride toxicity on metabolic changes, especially those associated with phosphorylation and dephosphorylation, in the liver and muscle of postnatal developing chicks, *Gallus gallus domesticus*.

MATERIAL AND METHODS

One day old female Rhode Island Red chicks were obtained and maintained as described in chapter 5. Six birds each from control and experimental groups were sacrificed by decapitation, on days 1, 5, 10, 20 and 30 after the commencement of experiment. For initial biochemical values six birds were sacrificed on the day of purchase. Left liver lobe and gastrocnemius muscle were quickly removed and pulverized in various ice-cold buffers for enzymes and protein assays.

Analytical Methods

Acetylcholinesterase was assayed using the colorimetric method of Ellman *et al.* (1961).

Na⁺-K⁺ ATPase was assayed according to the method of Post and Sen (1967). Inorganic phosphate released was estimated following the method of Fiske and SubbaRow (1925).

Alkaline and Acid Phosphatases were estimated according to the method of Linhardt and Walter (1963).

Total protein was estimated according to the method of Lowry *et al.* (1951).

RESULTS

Acetylcholinesterase activity

Liver: The average AChE activity in the liver of control birds fluctuated during the initial 10 days of post-hatched development. AChE activity however, increased steeply on day 20. Further increase in AChE activity was recorded on the last phase of experimental regimen. Compared to control chicks, the AChE activity in the experimental birds declined significantly ($p < 0.05$) by day 5 of fluoride administration (Table I). The difference in the decrease of AChE activity, between control and experimental birds, increases with prolonged fluoride intoxication (Figure 1).

Muscle: The intensity of AChE activity in the gastrocnemius muscle of control chicks was much less compared to that of liver. The toxicant exerted its adverse effect on AChE activity in the muscle only by day 20 of experimentation. Thereafter the AChE activity in experimental birds remained at a low level compared to that of control chicks (Figure 2).

Na⁺-K⁺ ATPase activity

Liver: The mean Na⁺-K⁺ ATPase activity in the liver of control birds fluctuated at different stages of experiment. As compared to control birds the experimental birds started showing signs of reduced Na⁺-K⁺ ATPase activity by day 10 of fluoride administration. More significant reduction in Na⁺-K⁺ ATPase activity was observed in the liver of experimental birds

on day 20 and 30 of fluoride intoxication (Figure 3).

Muscle: From table II it is clear that the $\text{Na}^+\text{-K}^+$ ATPase activity in the muscle of control birds increased initially and then decreased gradually till day 20 of experimental schedule. On day 30, however, an increase in $\text{Na}^+\text{-K}^+$ ATPase was apparent. Although the $\text{Na}^+\text{-K}^+$ ATPase activity in the muscle of experimental birds followed a similar course, compared to control chicks, significant reduction in enzyme activity was observed on days 10, 20 and 30 of fluoride administration (Figure 4).

Acid phosphatase activity

Liver: A gradual decline in acid phosphatase activity was observed in the liver of control chicks till day 5 of experiment. Thereafter, the average acid phosphatase activity increases till the termination of experiment. Fluoride administration brought down the liver acid phosphatase activity by day 20. Further reduction in acid phosphatase activity was observed in the experimental birds on day 30 of fluoride poisoning (Figure 5).

Muscle: Figure 6 shows the trend of acid phosphatase activity in the gastrocnemius muscle of growing chicks. Fluoride poisoning adversely affected the acid phosphatase activity by day 20 of experiment. Thereafter, compared to control chicks the acid phosphatase activity in the experimental birds remained at a low level (Table II).

Alkaline phosphatase activity

Liver: In the liver of control birds the mean alkaline phosphatase activity declined initially and then escalated rapidly till day 10 of experiment. Thereafter, however, the alkaline phosphatase activity diminished till the termination of experiment. The average alkaline phosphatase activity in the liver of experimental birds also followed a similar pattern. Never-

TABLE I : Effect of subacute NaF on enzymes related to glucose uptake or release in the liver of growing chick.

Parameters studied	Duration of Treatment (Days)									
	0	1	5	10	20	30	Exp	Con.	Exp	Con.
AChE (μ M Acetylthiocholine Hydrolysed/mg Protein/Minute)	0.0126 \pm 0.0007	0.0135 \pm 0.0009	0.0093 \pm 0.0009	0.0066 \pm 0.0007 ^{**}	0.0080 \pm 0.0006	0.0157 \pm 0.0006	0.0103 \pm 0.0009 ^{**}	0.0144 \pm 0.0007	0.0103 \pm 0.0009 ^{**}	0.0092 \pm 0.0009 ^{**}
Na ⁺ K ⁺ ATPase (μ g P Released/mg protein/10 min)	9.27 \pm 0.12	8.84 \pm 0.12	13.39 \pm 0.28	13.32 \pm 0.31	7.87 \pm 0.14	14.02 \pm 0.28	7.32 \pm 0.19 [*]	8.46 \pm 0.16	6.90 \pm 0.16 [*]	11.35 \pm 0.36 [*]
Acid phosphatase (μ M PNP Released/mg protein/30 min)	3.43 \pm 0.11	3.19 \pm 0.12	2.61 \pm 0.09	2.55 \pm 0.11	3.27 \pm 0.08	5.50 \pm 0.12	3.31 \pm 0.08	4.80 \pm 0.08	3.54 \pm 0.07 [*]	3.21 \pm 0.08 [*]
Alkaline phosphatase (μ M PNP Released/mg protein/30 min)	0.695 \pm 0.010	0.654 \pm 0.022	0.806 \pm 0.012	0.815 \pm 0.013	0.852 \pm 0.010	0.662 \pm 0.008	0.847 \pm 0.011	0.704 \pm 0.010	0.610 \pm 0.008 [*]	0.528 \pm 0.0140 [*]

@ Values are expressed as mean \pm SEM of six experiments. * p < 0.05; ** p < 0.02; *** p < 0.001

TABLE II : Effect of subacute NaF on enzymes related to glucose uptake or release in the muscle of growing chick.

Parameters studied	Duration of Treatment (Days)									
	0	1	5	10	20	30	Con	Exp	Con	Exp
AChE (μ M Acetylthiocholine Hydrolysed/mg Protein/Minute)	0.0054 \pm 0.0002	0.0061 \pm 0.0005	0.0060 \pm 0.0006	0.0047 \pm 0.0004	0.0039 \pm 0.0004	0.0040 \pm 0.0004	0.0033 \pm 0.0004	0.0072 \pm 0.0005	0.0045 \pm 0.0004	0.0045 \pm 0.0004 \downarrow
Na ⁺ -K ⁺ ATPase (μ g P Released/mg protein/10 min)	6.12 \pm 0.14	6.41 \pm 0.17	6.34 \pm 0.16	5.25 \pm 0.15	5.37 \pm 0.18	4.28 \pm 0.16	3.28 \pm 0.16 \downarrow	3.68 \pm 0.14	5.23 \pm 0.15	3.61 \pm 0.14 \downarrow
Acid phosphatase (μ M PNP Released/mg protein/30 min)	1.16 \pm 0.11	1.74 \pm 0.12	1.58 \pm 0.12	1.03 \pm 0.12	0.96 \pm 0.10	1.39 \pm 0.10	1.20 \pm 0.13	1.25 \pm 0.07	1.69 \pm 0.08	1.11 \pm 0.07 \downarrow
Alkaline phosphatase (μ M PNP Released/mg protein/30 min)	0.322 \pm 0.017	0.311 \pm 0.007	0.308 \pm 0.012	0.220 \pm 0.007	0.230 \pm 0.007	0.340 \pm 0.007	0.299 \pm 0.007 \downarrow	0.299 \pm 0.008	0.318 \pm 0.016	0.227 \pm 0.009 \downarrow

@ Values are expressed as mean \pm SEM of six experiments. ** p < 0.02; *** p < 0.01; **** p < 0.001

Fig. 1. Acetylcholinesterase Activity in Liver

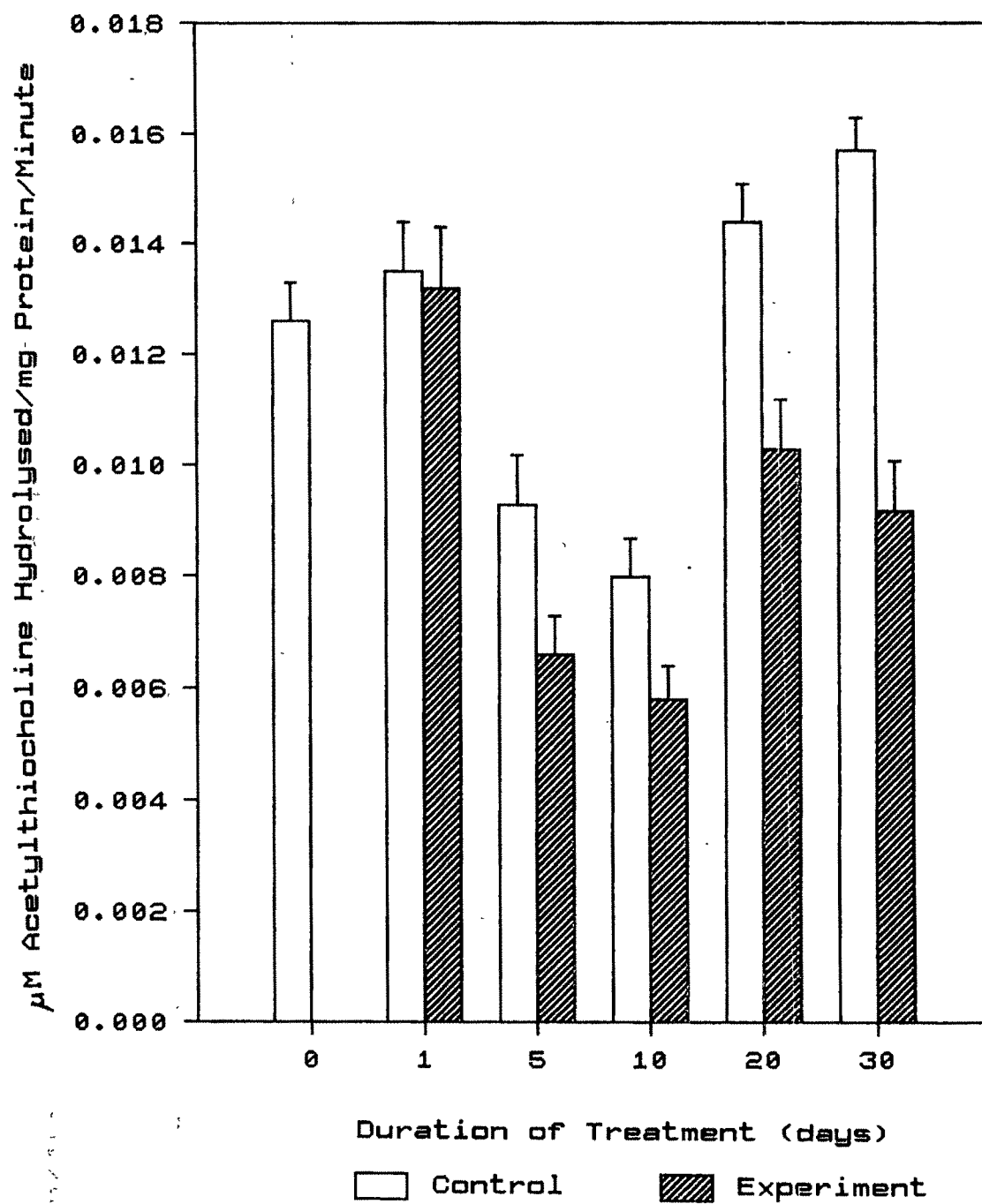


Fig. 2. Acetylcholinesterase Activity in Muscle

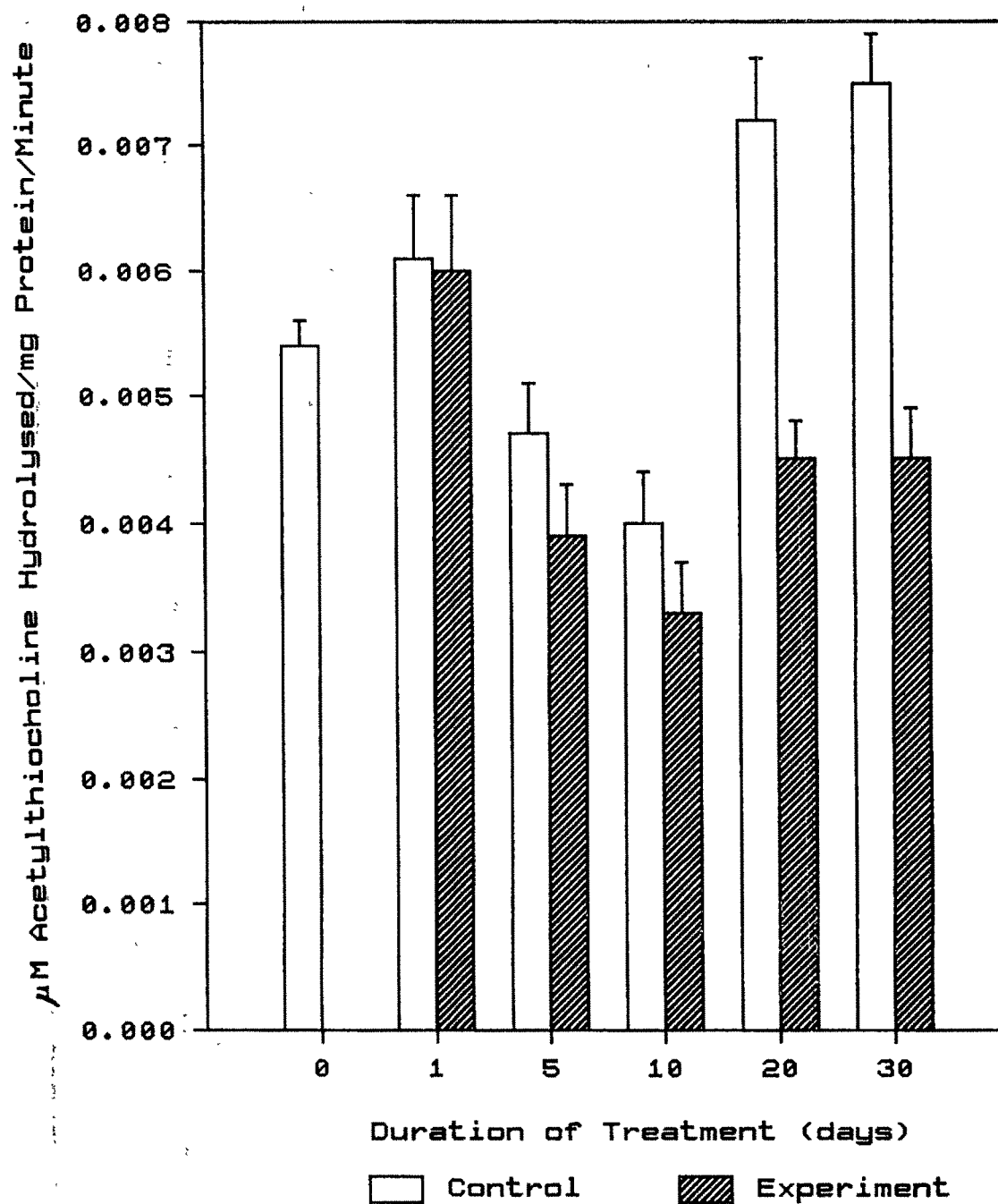


Fig. 3. $\text{Na}^+\text{-K}^+$ ATPase Activity in Liver

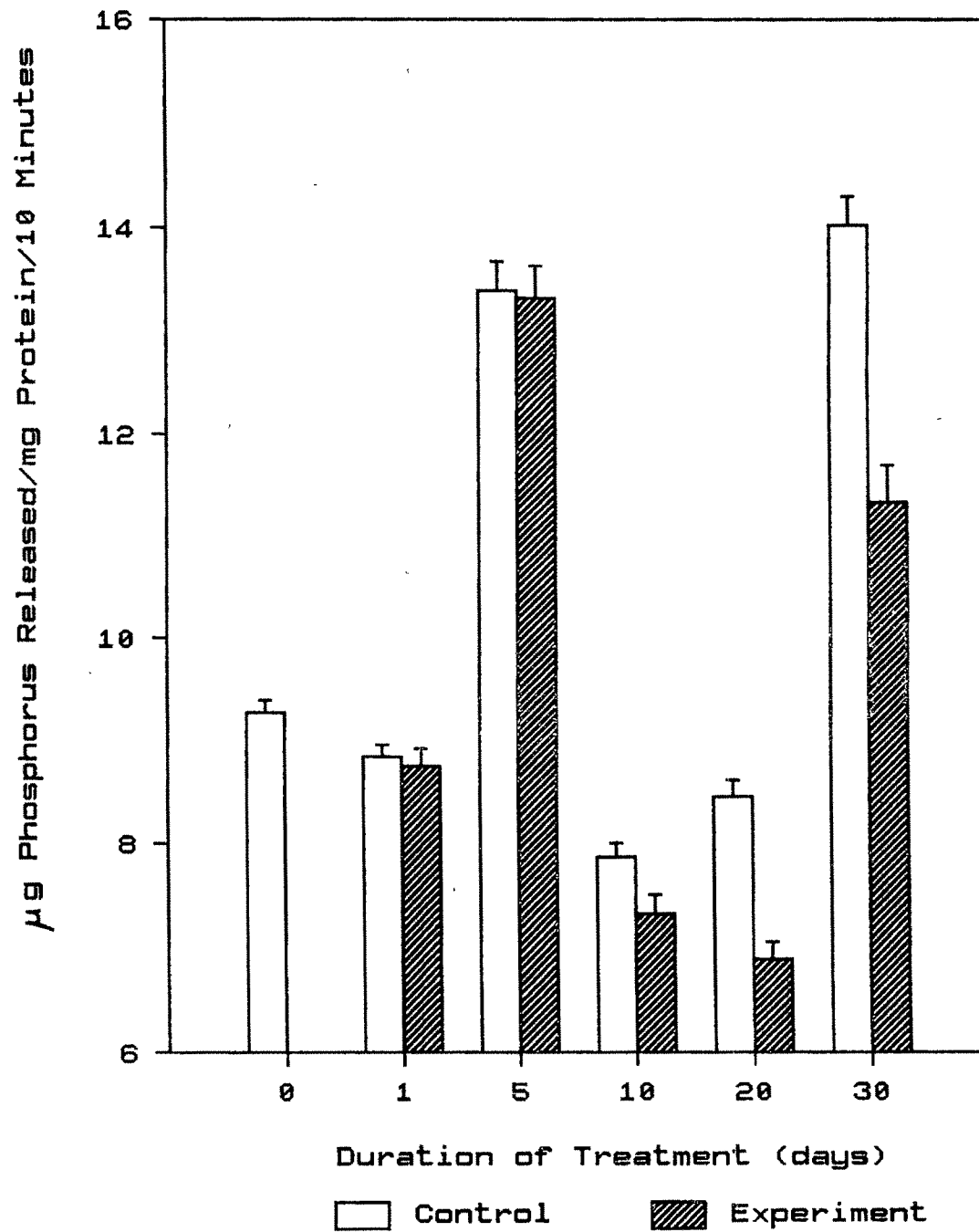


Fig. 4. $\text{Na}^+\text{-K}^+$ ATPase Activity in Muscle

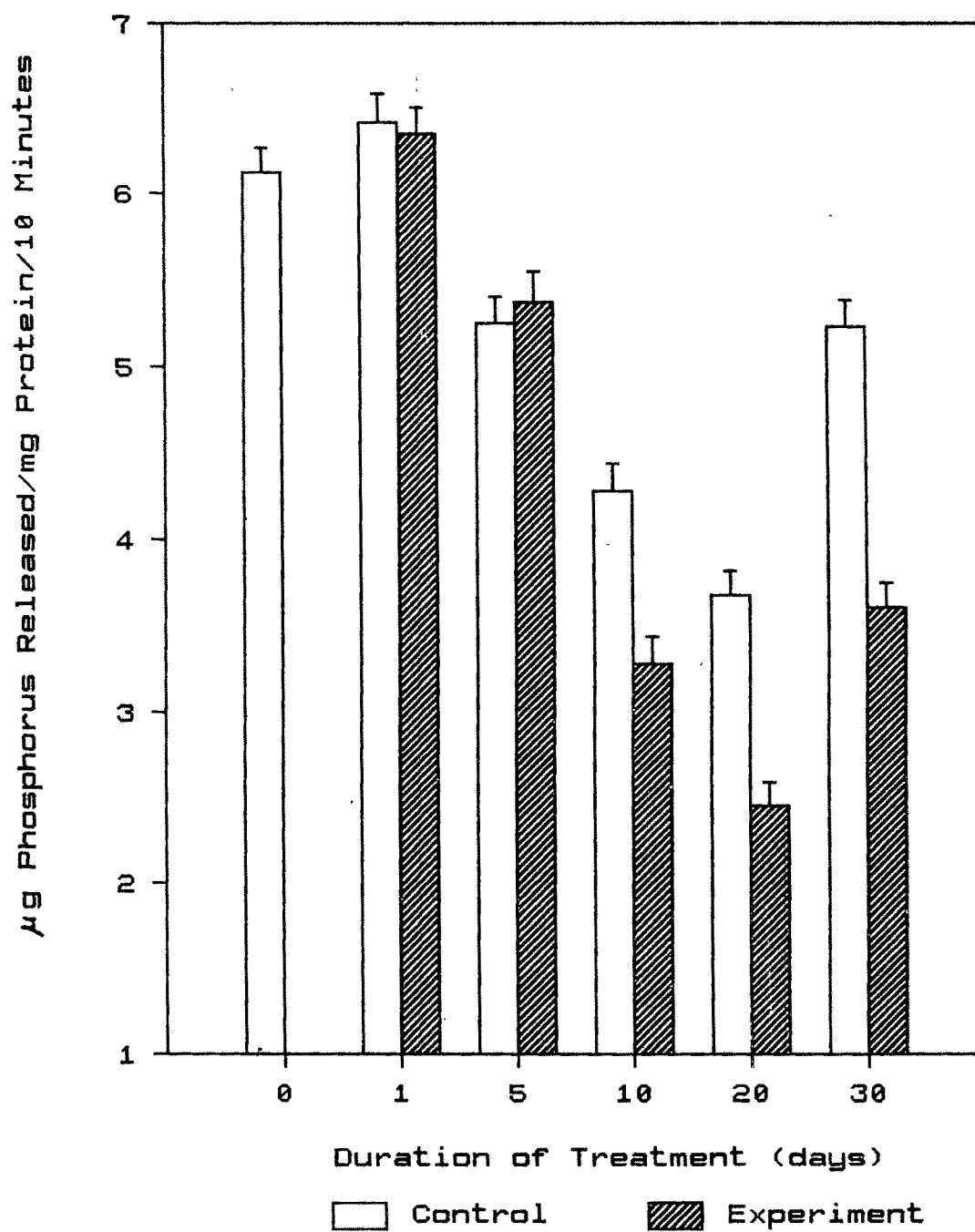


Fig. 5. Acid Phosphatase Activity in Liver

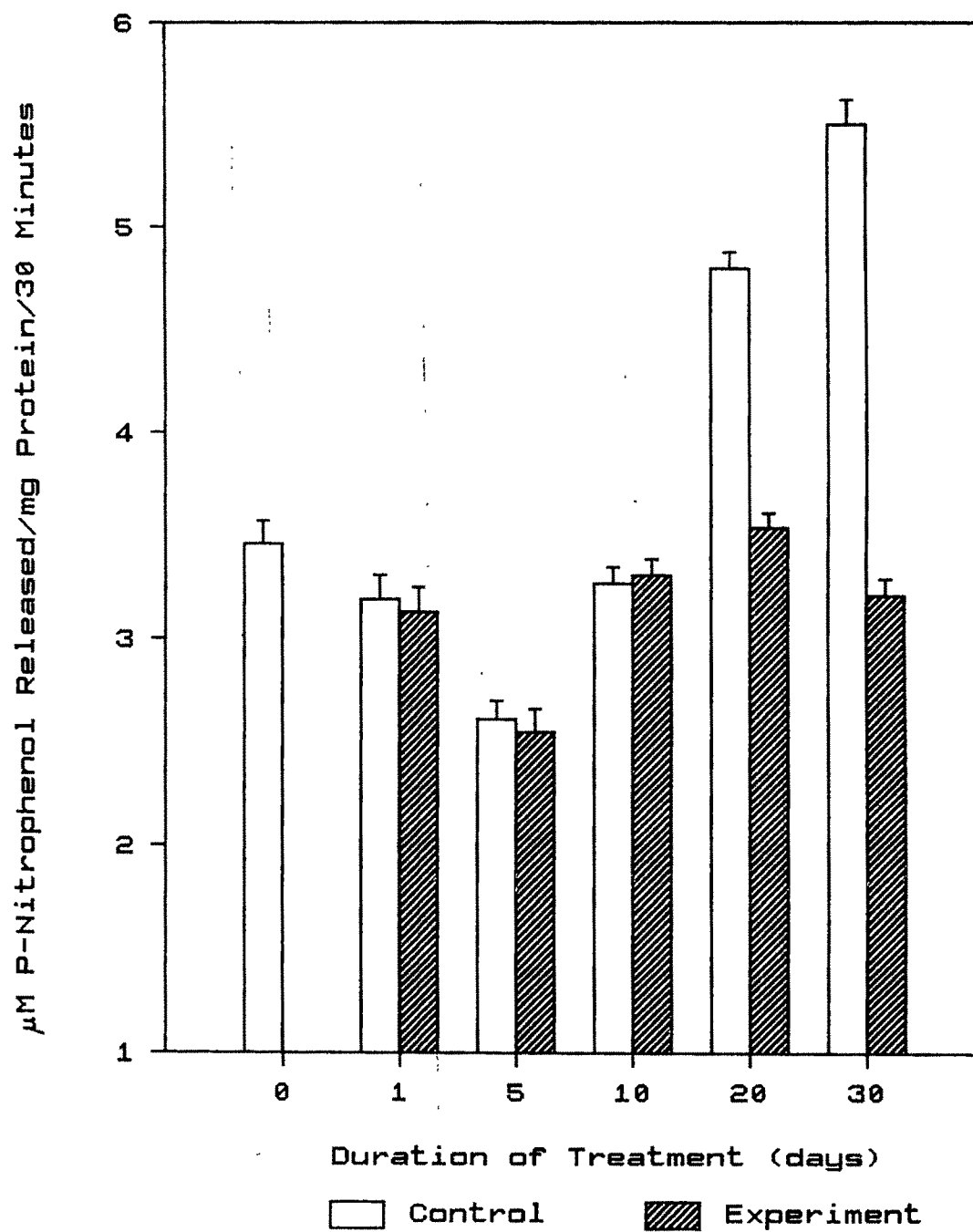


Fig. 6. Acid Phosphatase Activity in Muscle

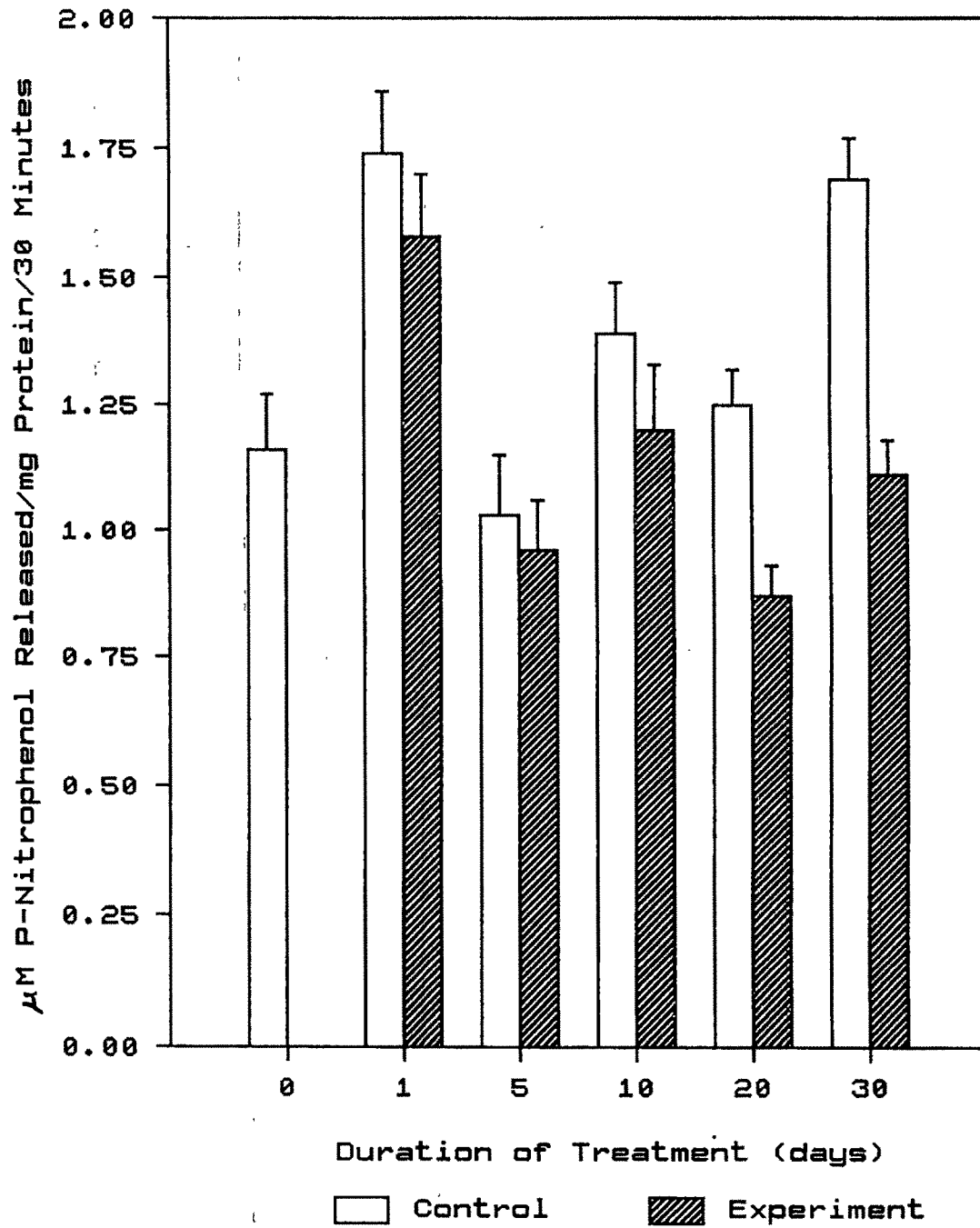


Fig. 7. Alkaline Phosphatase Activity in Liver

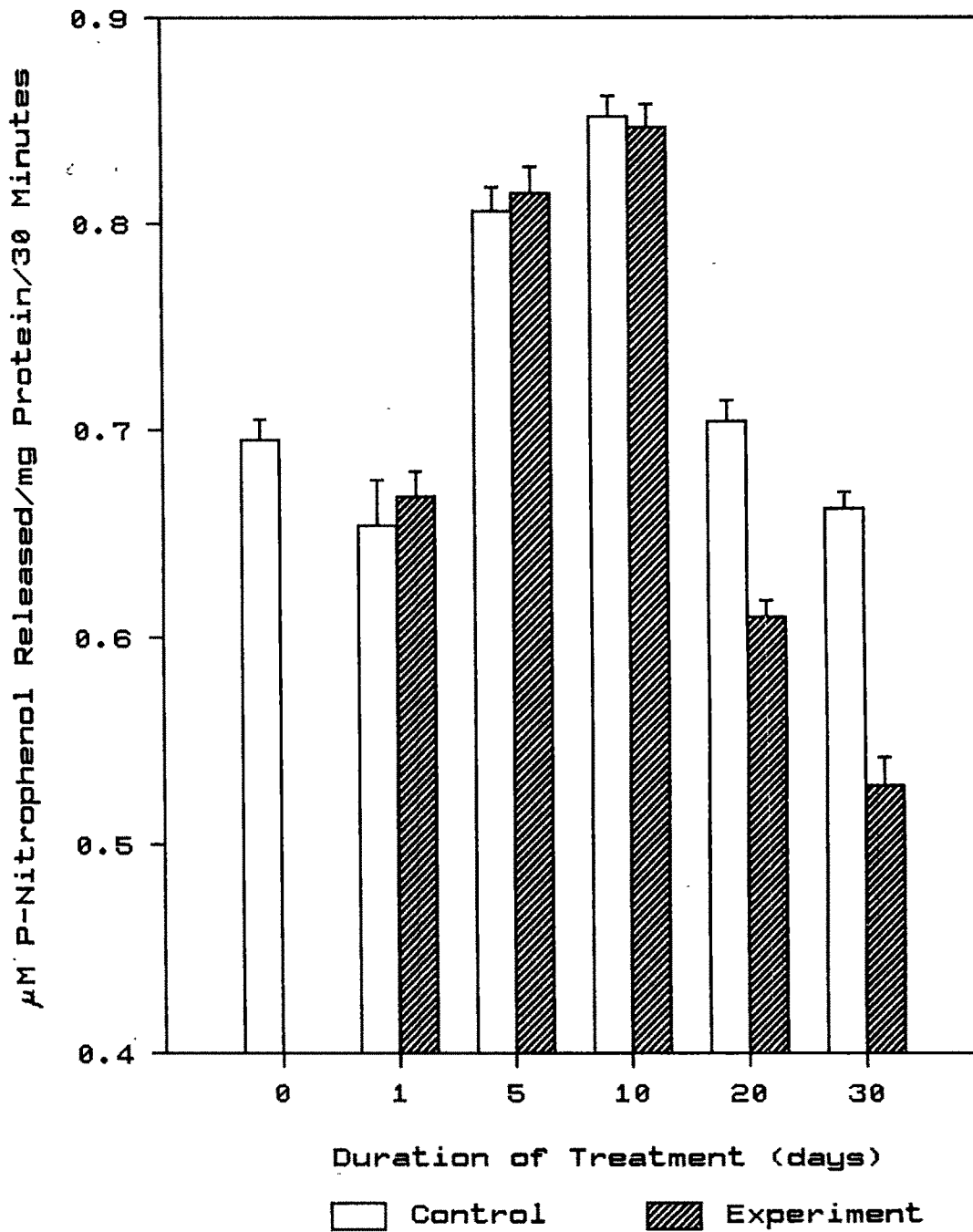
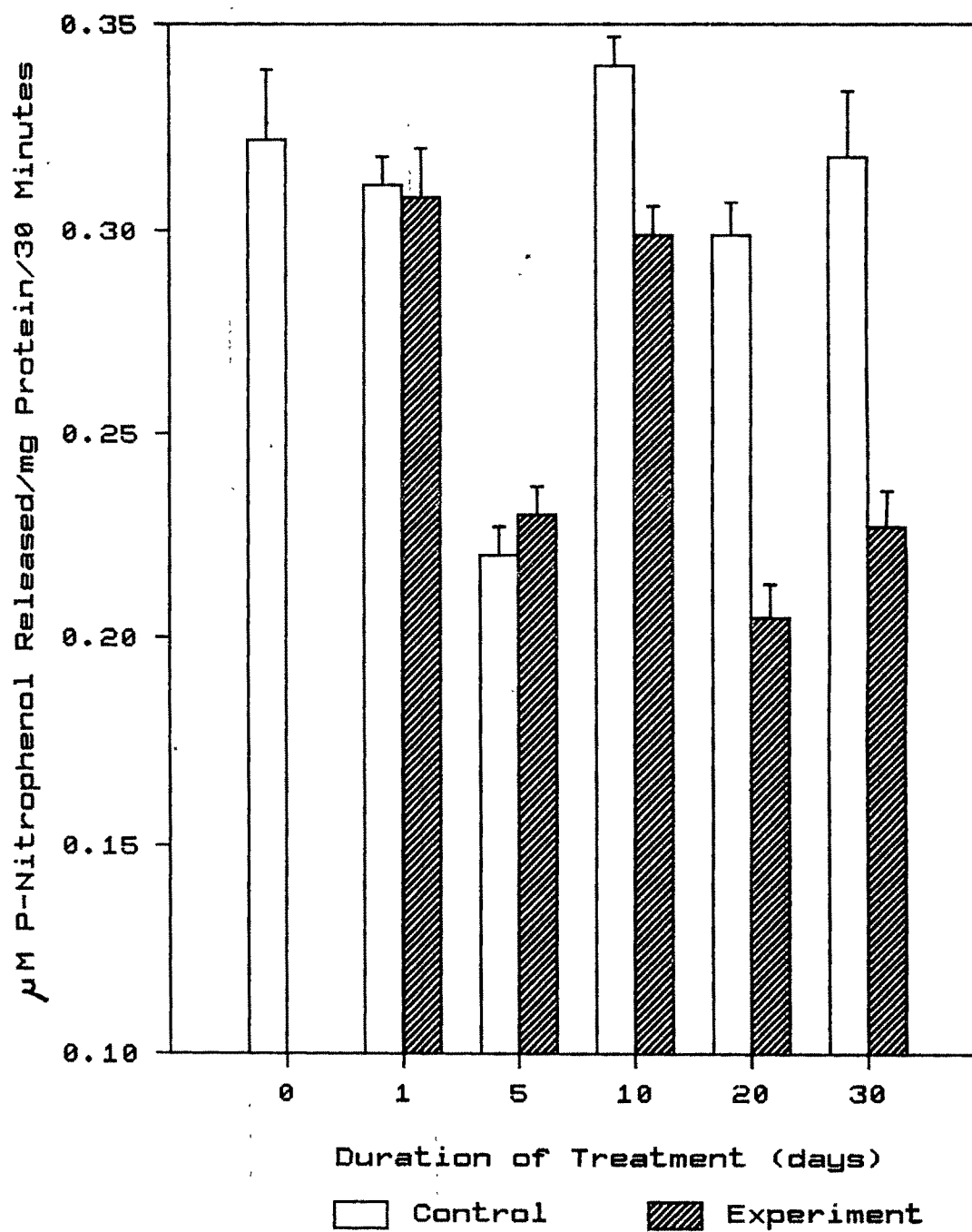


Fig. 8. Alkaline Phosphatase Activity in Muscle



theless, compared to control birds the enzyme activity in the experimental birds declined significantly ($p < 0.001$) by day 20. Further intoxication led to more reduction in alkaline phosphatase activity (Figure 7).

Muscle: The mean alkaline phosphatase activity in the gastrocnemius muscle of control chicks remained almost constant throughout the experimental regimen, except on day 5, where a drop in alkaline phosphatase activity was quite apparent (Table II). As compared to control birds the experimental birds registered a fall in alkaline phosphatase activity by day 10. Thereafter mean alkaline phosphatase activity in the experimental birds remained at a significantly low level till the end of experiment (Figure 8).

DISCUSSION

Chronic fluoride administration reduced the vagal influence in both the liver and the gastrocnemius muscle of growing chick. This was evident from the fact that AChE activity was very much reduced in the liver and muscle of experimental birds, when compared to that of controls. The degree of reduction in AChE activity increased with prolonged fluoride intoxication. It has been well documented that, several pesticides at subacute level cause inhibition of cholinesterase activity in different tissues (Sharma *et al.*, 1973; Quadri and Ahmed, 1979; Dikshith *et al.*, 1980; Sahib and Rao, 1980; Dutta *et al.*, 1994). The level of activity of AChE is an indicator of ACh secreted by the nerve endings (Pilo *et al.*, 1976) and ACh as well as insulin are known to assist in the uptake of glucose into the hepatic cells (Mondon and Burton, 1971; Pilo and Mehan, 1988). Not surprisingly, therefore, a dysfunction of cholinergic system often leads to hyperglycaemia (John *et al.*, 1985; Oommen, 1992). The fluoride intoxicated birds also showed an apparent hike in serum glucose level (Chapter 6). This adds strength to the present notion of a possible downregulation of vagal tone.

Both insulin and acetylcholine enhance glucose uptake through a membrane bound mechan-

ism, part of which is coupled to ionic movements (Pilo and Mehan, 1986; 1988). Na^+ and K^+ along with Ca^{2+} play a major role in membrane polarization and permeability by their differential distribution on either side of the cells. A considerable body of evidence indicates that active transport systems exist in avian intestine, that translocate amino acids and glucose against concentration gradients (Fearon and Bird, 1968). Any interference with co-existent Na^+ transport, reduces sugar transport as well, which indicates that the active component of the transport system may as well be that of Na^+ and that the sugar transport may be a secondary co-phenomenon (Alvarada and Monreal, 1967).

The cation concentration in the cell depends upon several factors such as permeability of cellular membrane, glycogen deposition as well as ionic concentrations in the extracellular fluid and blood. These factors are regulated by the transport enzyme $\text{Na}^+ - \text{K}^+$ ATPase. The presence of sodium pump in almost all animal cells has been demonstrated (Kaplan, 1983). This membrane bound enzyme is involved in the active transport of Na^+ and K^+ ions as well as essential metabolites like glucose and amino acids (Ganong, 1989).

In the present study a decrease in $\text{Na}^+ - \text{K}^+$ ATPase activity has been noticed in the liver and the gastrocnemius muscle of the fluoride treated chicks. Jain and Susheela (1987) have recorded reduced $\text{Na}^+ - \text{K}^+$ ATPase activity in the erythrocyte membrane of rabbits subjected to subacute sodium fluoride treatment for six months. Increased cyclic adenosine-3'5'-monophosphate (cAMP) concentrations are known to inhibit $\text{Na}^+ - \text{K}^+$ ATPase (Luly *et al.*, 1972; Barnabei *et al.*, 1973; Tria *et al.*, 1974) and it is well established that elevation in cAMP concentration accelerates glycogenolysis. From the previous chapter (Chapter 6), it is obvious that chronic fluoride administration accelerates glycogenolysis. This indicates a possible hike in cAMP turnover in fluoride treated birds. Five components are involved in the mechanism by which ligands bring about changes in the intracellular concentration of cAMP: a catalytic unit, adenylate cyclase which catalyses the conversion of adenosine

triphosphate (ATP) to cAMP; a stimulatory and inhibitory receptor and a stimulatory and inhibitory G protein that links the receptor to the catalytic unit. When an appropriate ligand binds to the stimulatory receptor, the α subunit of G_s activates adenylate cyclase. Conversely, when the appropriate ligand binds to the inhibitory receptor, the α subunit of G_i inhibits adenylate cyclase (Ganong, 1989). *In vitro* studies by Boyd *et al.* (1992), proved that sodium fluoride activates adenylate cyclase by direct interaction with $G_s \alpha$. An alternate explanation is that in case of hampered vagal tone (due to fluoride poisoning), sympathetic tone, which secretes catecholamines at their nerve endings, expresses in full and this could lead to increased formation of cAMP (Tomas *et al.*, 1970). Hence, it could be surmised that fluoride might have exerted its inhibitory effect on Na^+-K^+ ATPase through elevated cAMP level.

In the current study it was also observed that fluoride administration significantly lowered the acid and alkaline phosphatases activities in the liver and in the gastrocnemius muscle of growing chicks. Several studies involving pollutant effects on activities of acid and alkaline phosphatases have been carried out. Mukhopadhyay and Dehadrai (1980) showed that the activity of acid phosphatase was inhibited in the liver and gills of catfish exposed to 1 ppm malathion. Synergistic effects of phenol and dinitrophenol on acid and alkaline phosphatases were observed by Verma *et al.* (1980). Alkaline phosphatase in the gills of *Clarius batrachus* was inhibited in the event of cadmium toxicity (Banerjee *et al.*, 1978). The activity of alkaline phosphatase was noted to have been decreased in *Tilapia mossambica* exposed to sublethal concentration of monocrotophos (Joshi and Desai, 1981). Alkaline phosphatase activity in kidney, liver and muscles of a fresh water teleost was significantly inhibited after the exposure to rogor (Bhatnagar *et al.*, 1984).

There have been some reports on fluoride effects on acid and alkaline phosphatases activities. A decrease in Mg^{2+} dependent enzyme such as alkaline phosphatase and certain esterases in the serum of rats given water containing 100 ppm F^- for 50 days was reported (Riekst-

niece *et al.*, 1965). Inhibition of acid phosphatase by fluoride in human saliva and prostate gland was observed by Smith *et al.* (1950). Acid phosphatase is particularly fluoride sensitive as recorded by Befanti *et al.* (1935) and Kutscher and Wust (1942). This was further confirmed by Takagi and Shiraki (1982). They histochemically showed that the acid phosphatase is among the highly impaired enzymes in the early stages of acute toxic sodium fluoride neuropathy.

Mechanism of inhibition by fluoride on several enzyme systems has been investigated using fluoride concentration, several times higher than that present in normal body fluids. Fluoride can partly bring about enzyme inhibition by being absorbed on (and thus blocking) the active sites of the enzyme required for the formation of enzyme-substrate complex (Shaikh, 1985). The inhibition of alkaline phosphatase activity may be due to uncoupling of phosphorylation. Similar suggestion is made by Bhatnagar *et al.* (1984), while studying the effect of rogor on fish. The inhibition of the activity of the enzyme in liver indicates that the transphosphorylation reactions are adversely affected in this organ. As the liver is the main detoxification organ in the body, maximum damage to the physiological functioning is caused by fluoride as revealed by inhibition in the activity of acid and alkaline phosphatases.