CHAPTER 2

HISTOCHEMICAL DEMONSTRATION OF ALKALINE AND ACID PHOSPHATASES IN THE MAMMALIAN DIAPHRAGM

Alkaline phosphatase has been histochemically demonstrated in various tissues. It has been demonstrated in the developing tissues where the activity of enzyme is high (Rogers, 1963). Its activity has also been demonstrated in the various tissues of the regenerating tail of the wall lizard Hemidactylus flaviviridis (Shah and Chakko, 1967). However its demonstration in the skeletal muscle has not been fully successful. Dempsey et al.,(1946), Newman et al., (1950), obtained a negative reaction for alkaline phosphatase in the skeletal muscle fibres. It has also been reported to be absent in human foetal muscle and the adult human muscle (Rossi et al., 1954, Mackay et al., 1955, Beckett and Bourne, 1958). A positive staining reaction was obtained in the pigeon breast muscle after prolonged incubation of the muscle sections (George, Nair and Scaria, 1958). Rudolph (1959) working on the diaphragm could obtain a positive reaction only in the blood capilliaries in the muscle. Recent attempts of Ogata and Mori (1963) employing the azo-dye coupling technique however failed to demonstrate alkaline phosphatase in the skeletal muscles.

Similarly acid phosphatase was claimed to be absent in the skeletal muscles. The histochemical localization of this enzyme was successfully demonstrated in the pectoralis of pigeon by using Glick's method (George and Pishawikar, 1961). Recently Ogata and Mori (1963) failed to demonstrate this hydrolytic enzyme in the skeletal muscles of various animals. As against this, number of workers demonstrated its presence in the skeletal muscles of various animals. Greenstein (1942) demonstrated its presence in the mouse skeletal muscles. Vallyathan and George (1965) demonstrated its presence in the pectoralis of pigeon.

In the light of these observations it was thought desirable to investigate further on the possibilities of demonstrating the alkaline and acid phosphatase activities in the muscle and mammalian diaphragm was chosen as the material.

MATERIALS AND METHODS

In the case of small animals the diaphragm was collected immediately after decapitating them. When the diaphragm of larger mammals was to be used, the animals were killed by cutting the jugal vein and the diaphragm was collected immediately. After the removal of the diaphragm, it was blotted well to remove blood and was

spread on clean dry filter paper and three regions (dorsal, lateral and ventral) were separated as described by George and Susheela (1961). After separating the three different regions fresh frozen sections about 20 μ thick were cut from each of the three regions separately and treated for histochemical demonstration of alkaline and acid phosphatases by employing the following techniques.

Alkaline phosphatase

For the histochemical demonstration of alkaline phosphatase the diaphragms of rat, rabbit, goat, cat, civet cat, dog and monkey were used.

The fresh frozen transverse sections were spread on clean dry slides and placed in the ice cold 10% neutral formalin (neutralized with $NaHCO_3$) and fixed for two hours. After fixation they were washed under running tap water and repeatedly rinsed with distilled water to remove formalin completely. The slides were then incubated for 20 hours in a medium containing sodium-β-glycerophosphate as the substrate at 37°C. The pH of the incubation medium was maintained at 9.0. The incubation medium contained the following ingredients (Pearse, 1954 Gomori's method).

2% Sodium-β-glycerophosphate	25 ml.
2% Sodium barbitone	25 ml.
2% Calcium chloride	05 ml.
5% Magnesium Sulphate	02 ml.
Distilled water	50 ml.

Two sets of controls were run, one set of slides were treated with hot water (90°C) for 15 minutes prior to incubation and the other set was incubated in the medium without the substrate.

After incubation the slides were rinsed with distilled water and then treated with 2% cobalt nitrate solution for 5 minutes. The slides were thoroughly washed with distilled water. They were then treated with dilute yellow ammonium sulphide with which the cobalt ions react to form black deposits of cobalt sulphide, at the sites of the enzyme activity. The slides were then rinsed with distilled water and mounted in glycerine jelly.

It was found that a higher concentration of magnesium sulphate in the incubation medium was essential for activating the enzyme. Various concentrations of the salt were tried and it was found that 5% $MgSO_4$ in the medium gave satisfactory results.

Acid phosphatase

For histochemical demonstration of acid phosphatase the diaphragm of the mammals like rat, rabbit, cat, dog, civet cat and monkey were used.

The fresh frozen transverse sections were spread on the clean dry slides. The tissue sections were fixed for 2 hours in ice cold 10% neutral formalin (neutralized with

$NaHCO_3$). The sections were then washed under running tap water and rinsed well with distilled water to remove the fixative completely. The sections were then stained by lead-sulphide method for the histochemical demonstration of acid phosphatase (Glick, 1949 as described by George and Pishawikar, 1961). In this method the sections were incubated in an incubation medium containing sodium-β-glycerophosphate and lead nitrate. The pH of the incubation medium was kept at 5.2. The sections were mounted in glycerine jelly. The brown black precipitates are formed at the sites of enzyme activity.

OBSERVATIONS

Alkaline phosphatase was found to be localized in the sarcoplasmic reticulum in all the cases. It was observed that in all the animals studied the intensity of enzyme activity was the same in the tonic as well as in the phasic fibres. The staining was also intense in the periphery of the fibre in the region of the sarcolema in all the cases. The distribution pattern of the enzyme activity in all the three regions of the diaphragm was also similar (Figs.1, 2 and 3).

Acid phosphatase was found to be present in all the three types of fibres in all the regions and in all the animals studied. It did not follow the same distribution

pattern as that of the enzymes like SDH and lipase. The lateral region showed a slightly higher activity of acid phosphatase in all the cases studied. In the diaphragm of the smaller animals the enzyme activity was found to be higher as compared to that of the larger ones (Fig. 4).

Table 1

Showing the level of acid phosphatase activity as determined on the basis of the period of incubation in hours.

Animal	Dorsal region	Lateral region	Ventral region
Rat	4	3	4
Rabbit	5	3	6
Cat	5	4	5
Dog	6	6	8
Civet cat	6	5	6
Monkey	7	7	8

DISCUSSION

The activities of alkaline and acid phosphatases have been correlated with various functions of the cell viz. Protein synthesis (Wolf et al.,1943; Kivalo, 1958), active transport of material (Moog, 1946; Romanul and Bannister,

1962) and activity of pinocytotic apparatus and secretion vacuoles (Barka, 1962). Although such functions are attributed to these enzymes, their possible role in transphosphorylation is well supported.

From the present observations, it is evident that alkaline phosphatase activity is mainly confined to the sarcoplasmic reticulum of the muscle fibre. It has been recently demonstrated by Padykula and Gautheir (1963) that ATPase activity is also located in the sarcoplasmic reticulum in addition to mitochondria in the rat diaphragm. They have also shown that the sarcoplasmic reticulum in the broad phasic fibres is more prominent than that of the narrow tonic fibres. However, in the present observations on the diaphragm of different mammals no distinct differences between the pattern of the enzyme activity in the different types of fibres could be seen. The most intense and distinct staining reaction for the enzyme activity was obtained in the dog diaphragm.

The sarcotubular location suggests that the metabolites in the sarcoplasm are transported through the mediation of this enzyme as in the proximal tubules of the kidney or in the mucosa of small intestine.

Recent studies on the Histochemical localization of alkaline and acid phosphatases in the pigeon breast muscles

by Vallythan and George (1965) demonstrated both the above mentioned enzymes in the sarcoplasmic reticulum of the muscle fibres. However, in the present observations on the histochemical demonstration of acid phosphatase, it was found that in the muscle fibres of diaphragm the enzyme is localized in the cytoplasm and not in the sarcoplasmic reticulum as in the case of alkaline phosphatase. The study of acid phosphatase was made in the diaphragm of mammals of various sizes. It was found that the said enzyme activity can be demonstrated in a shorter period of incubation in the smaller mammals as compared to the larger forms. The acid phosphatase was found to be equally distributed in all the types of fibres. The enzyme activity was found to be more in the lateral region as compared to the two other regions. The high level of this enzyme in the lateral region and in the smaller mammals can be correlated with the activity of the region which is greater than the other two regions, as well as its high level in the smaller mammals with the higher respiratory rate. The enzyme acid phosphatase is also said to be playing an important role in the transport of metabolites by way of transphosphorylation.

Vallythan and George (1965) suggested that the presence of alkaline and acid phosphatases in the sarcoplasmic reticulum can be regarded as indicative of the function of these

enzymes in the transport of glycogen to the interior of the cells from the sites of its synthesis. Khan and George (1967) found that the alkaline and acid phosphatases are localized in the mitochondria while the sarcoplasmic reticulum was found to be faintly stained. The eletron microscopic study of Clark (1961) on the rat diaphragm has also shown the localization of alkaline phosphatase activity in the sarcoplasmic reticulum. Varrati (1902) also suggested the localization of both these enzymes in the sarcoplasmic reticulum. All the above workers agree with the fact that both the enzymes are localized in the sarcoplasmic reticulum. Moreover the observations on the localization of ATPase (Bokdawala and George, 1965) and Glucose-6-Phosphate dehydrogenase (Nene and George, 1965) in the sarcoplasmic reticulum have revealed the importance of this system in the metabolism of muscle fibres. These findings aid to conclude that both alkaline and acid phosphatases play an important role in the intracellular transport of metabolites.

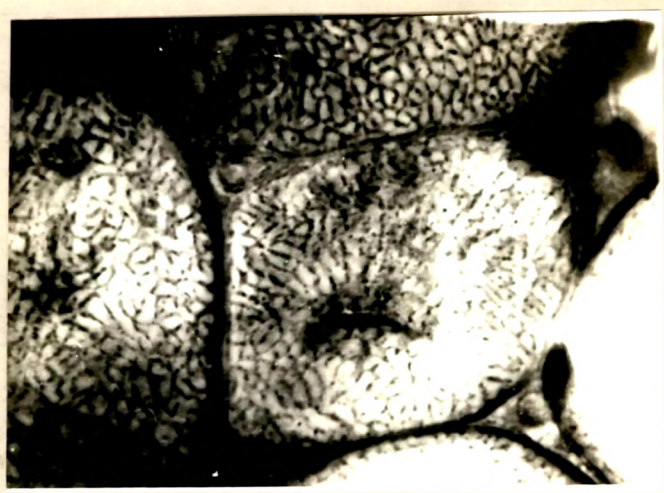

Fig. 1

Photomicrograph of the transverse section of the dog diaphragm showing the localization of alkaline phosphatase activity in the sarcoplasmic reticulum. 800 x

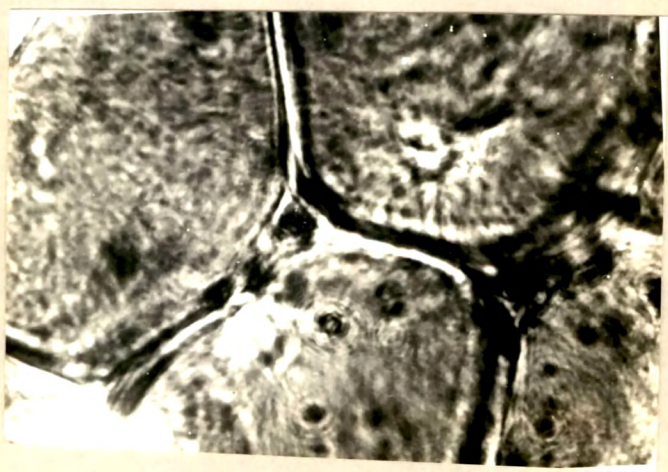

Fig. 2

Photomicrograph of the transverse section of the Goat diaphragm showing the localization of alkaline phosphatase activity in the sarcoplasmic reticulum. 800 X

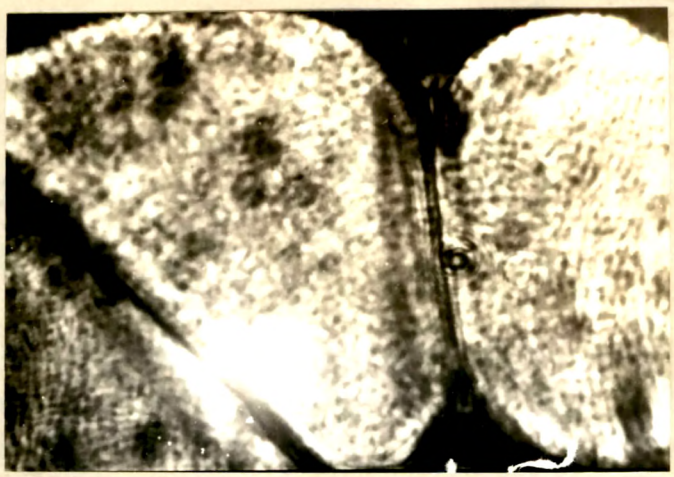

Fig. 3

Photomicrograph of the transverse section of the Civet cat diaphragm showing the localization of alkaline phosphatase activity in the sarcoplasmic reticulum. 800 X

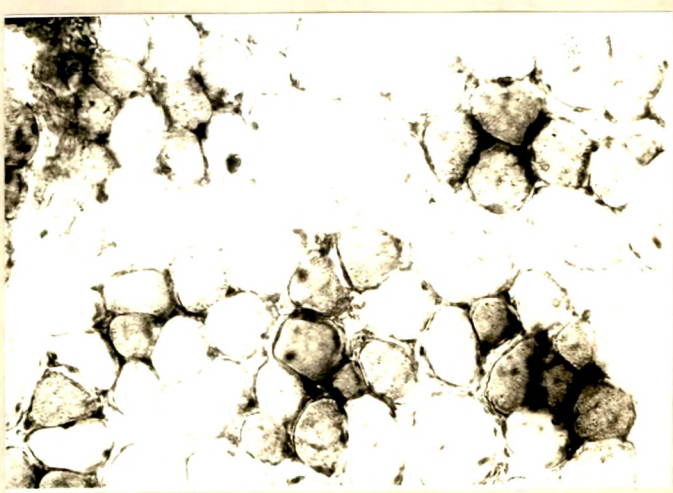

Fig. 4

Photomicrograph of the transverse section of the Civet cat diaphragm showing the localization of acid phosphatase activity in the sarcoplasm. No marked difference in the distribution pattern of the enzyme activity in different types of fibres is seen. 128 X.