

CHAPTER 3

HISTOCHEMICAL DEMONSTRATION OF CHOLINESTERASES
IN MAMMALIAN DIAPHRAGM

It is an established fact that the diaphragm is a mixed muscle consisting of the white, glycogen-loaded and the red, fat-loaded fibres (George and Susheela, 1961). It may be considered as occupying an intermediate position with respect to activity between the constantly contracting heart and intermittently contracting appendicular muscles.

It is also known that the two types of muscle fibres of a mixed muscle not only differ in their histochemical nature but they also differ in the nature of their neuromuscular junctions which innervate them. Krüger (1952, 1958) described the fast contracting fibres as possessed by "en plaque" type of nerve-endings, and the slow contracting fibres the "en grappe" type of nerve-endings. From the work of Häggqvist (1960, 1962), it is also known that acetyl cholinesterase (AChE) is found at "en plaque" type of nerve-endings, and the butyryl cholinesterase (BuChE) at "en grappe" type of nerve-endings. This kind of morphological difference of the nerve-endings was also shown by Zenker and Anzenbacher (1964) on their studies of the two types of muscle fibres from the external ocular muscles of Rhesus monkey. Klinar and Župančić (1962) worked on the two types of skeletal muscles of mammals like cat and rat and demonstrated that both the types of

cholinesterases are present at every nerve-ending irrespective of the type of fibres. Chinoy and George (1965) studied cholinesterases in the pectoralis major muscles of various vertebrates and showed that in all the vertebrates investigated, the morphological structure of the nerve-endings was of "en plaque" type.

In the light of above observations it was thought desirable to study the histochemistry of cholinesterases and the morphology of neuromuscular junctions in the mammalian diaphragm.

MATERIALS AND METHOD

In the case of small animals the diaphragm was collected immediately after decapitating them and the larger mammals were killed by cutting the jugal vein and the diaphragm was excised immediately. The diaphragms from various mammals like rabbit, rat, squirrel, hedge hog, dog, cat, fox and monkey were studied. After the removal of the diaphragm it was blotted well to remove blood and was spread on a clean filter paper and the three regions (dorsal, lateral and ventral) were separated as described by George and Susheela (1961). After separating the three regions, fresh frozen longitudinal sections, about 20 μ thick were taken from each of the three regions separately. The sections were fixed in ice cold, 10% formol saline

(Gurr, 1956) for one to two hours at 4°C. After fixation the sections were washed thoroughly with distilled water to remove the formalin completely. The activity of cholinesterases was studied by using the modified method by Coupland and Holmes (1957) of Koelle and Friedenwald (1949). The muscle sections of all the three regions were incubated separately in order to demonstrate the two enzymes (AChE and BuChE), Acetyl thiocholineiodide and butyryl thiocholineiodide were employed as substrates for AChE and BuChE respectively. The sections were incubated at 37°C at a pH varying between 5.6 to 6.0. The enzyme activity was judged by the length of the period of incubation and the intensity of the enzyme reaction.

RESULTS

The results obtained are presented in Table 1.

(1) Though the diaphragm is a mixed muscle consisting of two basic types of fibres (George and Susheela, 1961) or three types of fibres as described in chapter 1 (Figures of Chapter 1), in all the types of the muscle fibres and in all the three regions of the diaphragm of all the mammals investigated, the neuromuscular junctions were found to be of "en plaque" type (Figs. 1 to 6).

(2) AChE and BuChE were found to be present at the same nerve-endings.

Table 1

Activity of Cholinesterases in Mammalian Diaphragm

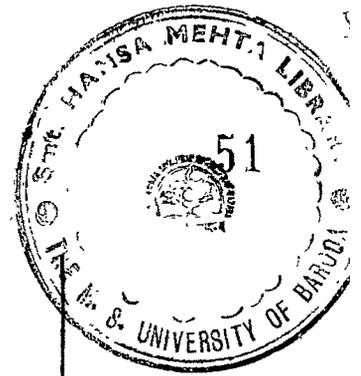
| Mammals investigated | Hours of incubation | | | | Activity of the enzymes | | | | | | | |
|----------------------|---------------------|-------|----------------|-------|-------------------------|-------|---------------|-------|----------------|-------|----------------|-------|
| | Dorsal region | | Lateral region | | Ventral region | | Dorsal region | | Lateral region | | Ventral region | |
| | AChE | BuChE | AChE | BuChE | AChE | BuChE | AChE | BuChE | AChE | BuChE | AChE | BuChE |
| Rabbit | 24 | 24 | 24 | 24 | 24 | 24 | + | + | + | + | + | + |
| Rat | 4 | 24 | 4 | 22 | 4 | 21 | ++++ | + | ++++ | + | ++++ | + |
| Squirrel | 4 | 5 | 4 | 4 | 4 | 5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Hedge hog | 3 | 4 | 3 | 4 | $3\frac{1}{2}$ | 5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Dog | 16 | 18 | 16 | 18 | 16 | 18 | ++ | ++ | ++ | ++ | ++ | ++ |
| Cat | 11 | 18 | 10 | 19 | 11 | 13 | +++ | ++ | +++ | ++ | +++ | +++ |
| Fox | 21 | 21 | 21 | 21 | 21 | 21 | + | + | + | + | + | + |
| Monkey | 24 | 24 | 24 | 24 | 24 | 24 | + | + | + | + | + | + |

3 to 5 hours ... maximum activity ... +++
 6 to 15 hours ... moderate activity... +++
 16 to 20 hours ... low activity ... ++
 20 to 24 hours ... least activity... +

Contd.....

Table 1 (Contd.)

| Mammals investigated | Diameter of the Nerve endings in terms of 'u' | | | | | |
|----------------------|---|-------|----------------|-------|----------------|-------|
| | Dorsal Region | | Lateral Region | | Ventral Region | |
| | AChe | BuChe | AChe | BuChe | AChe | BuChe |
| Rabbit | 30 | 30 | 30 | 30 | 30 | 30 |
| Rat | 35 | 25-30 | 35-40 | 25-30 | 20-25 | 20 |
| Squirrel | 25 | 25 | 30-35 | 25-30 | 25 | 25 |
| Hedge hog | 25-30 | 25-30 | 35-50 | 36-40 | 25-35 | 25-30 |
| Dog | 25-30 | 25 | 25-35 | 20-25 | 20 | 15-20 |
| Cat | 30-35 | 15-25 | 35-50 | 30-35 | 15-20 | 20 |
| Fox | 15-25 | 15-25 | 20-25 | 15-25 | 15-25 | 25-35 |
| Monkey | 15-20 | 15-20 | 20-25 | 20-25 | 10-15 | 20-25 |



(3) AChE was found to be higher than BuChE in the three regions of the diaphragm of all the mammals studied.

(4) Usually the lateral region showed the highest activity of cholinesterases than the other two regions.

(5) The intensity of the activity of cholinesterases was found to be inversely proportional to the body size of the animal, as revealed by the period of incubation. Smaller the animal greater the activity of both the cholinesterases.

(6) From Table 1 it is clear that the area of the nerve-endings is larger in smaller mammals as compared to the mammals bigger in size.

(7) Based on the intensity of the enzyme activity the mammals studied can be divided into four groups. I. Rat - Very high AChE activity as compared to BuChE activity. II. Squirrel and Hedge hog - High activity of both the enzymes. III. Cat and Dog - Low activity of both the cholinesterases. However, in cat AChE was moderate. IV. Rabbit, Fox and Monkey forms the fourth group in which both the types of cholinesterases seemed to be in equal concentrations, the least as compared to the other three groups.

(8) As seen from the diameter of the nerve-endings rat, hedge hog, squirrel, cat and rabbit can be grouped under one head where the contact surface is more as

compared to the other group consisting of dog, fox and monkey.

DISCUSSION

Both AChE as well as BuChE activities were found to be present at all the nerve-endings in both the types of fibres though their concentrations differed. No relationship was found between the type of nerve-ending and the type of muscle fibre, as it was previously described by Krüger (1952, 1958). All the nerve-endings were found to be of "en plaque" type only, as it was recently described by Chinoy and George (1964) in the pectoralis major muscle of various vertebrates.

Though the diaphragm is a mixed muscle the distribution pattern of the two types of fibres did not show any definite arrangement. The regional difference of three different regions of the rat diaphragm namely the dorsal, lateral and ventral were studied by George and Susheela (1961). They have shown that the lateral is specialized for carbohydrate metabolism as well as capable of oxidizing fatty acids more rapidly than the other two regions. Beck and Baxter (1960) have also shown that amongst the three different regions, the lateral has the major blood supply. Chinoy and George (1964) have also shown AChE to be more in the muscles having sustained activity. In the present investigation the high

enzyme activity and greater area of nerve-endings were found in the lateral region of the diaphragm as compared to the other two regions, suggesting the relationship between the sustained activity of the region and the higher concentration of both the cholinesterases.

From the work of Krebs (1950) it is evident that the basal metabolic rate per unit body weight is inversely proportional to the size of the animals. Even the number of breaths per minute was also found to be decreasing with the increasing body weight (Gessel and Atkinson, 1943). The results obtained in the present work showed that the intensity of these enzymes was greater in smaller mammals than the bigger ones. Thus the high enzyme activity of these enzymes in smaller animals like rat, hedge hog, and squirrel corresponds to their higher respiratory rate. The bigger mammals like dog, fox and monkey showed low activity as they have low respiratory rate.

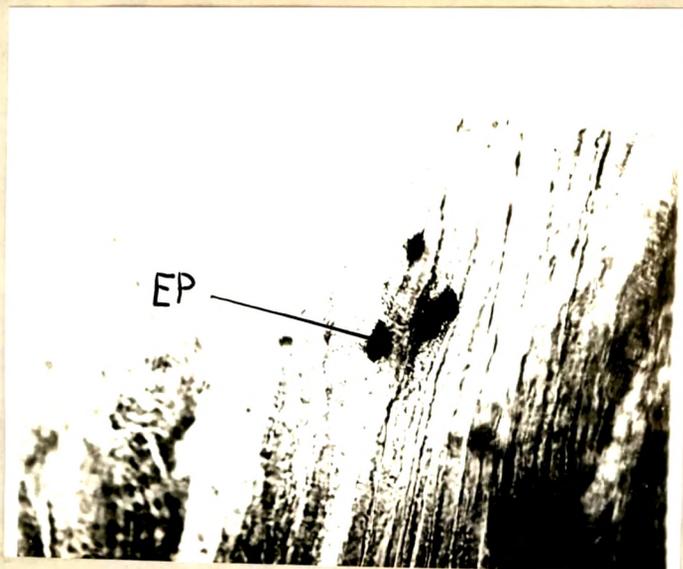


Fig.1

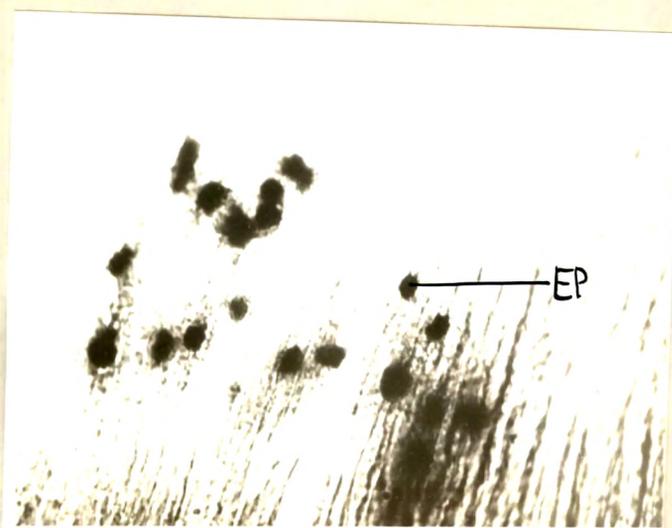


Fig.2

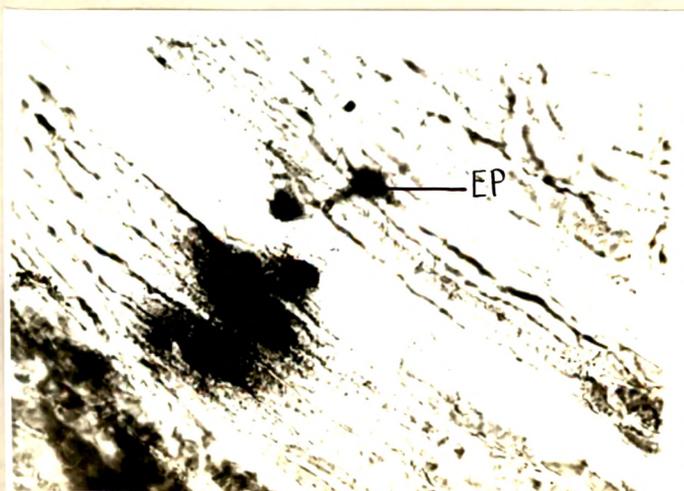


Fig.3

Figs.1,2, & 3
 Histochemical
 demonstration of
 Acetyl cholinesterase in the
 Diaphragm of Fox. Muscle sections
 (longitudinal) stained for the
 localization of AChE exhibit the
 "en plaque" type of nerve-endings.
 Figs. 1, 2 & 3 are for dorsal,
 lateral and ventral regions respectively.
 Nerve-endings are marked with 'EP'.
 All 128x.

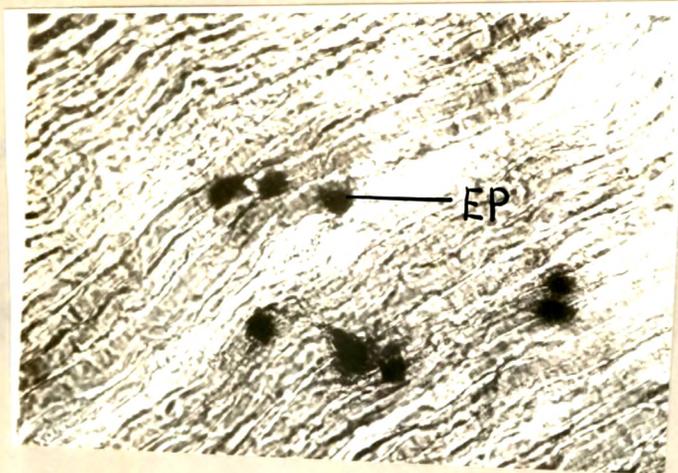


Fig.4

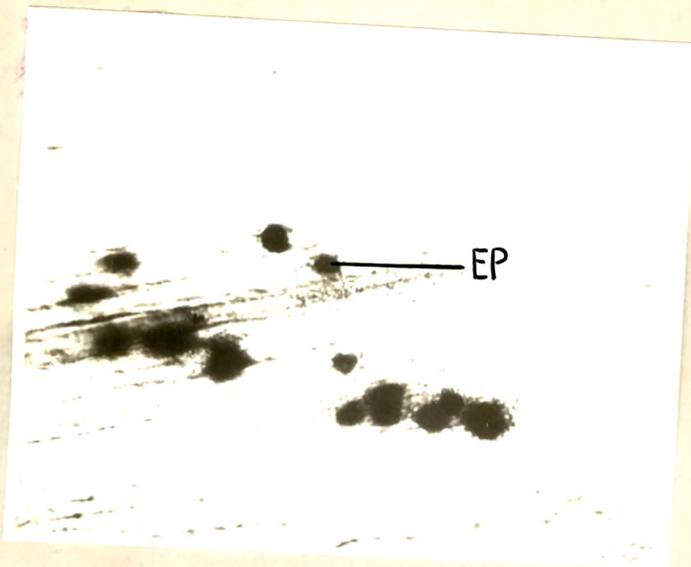


Fig.5

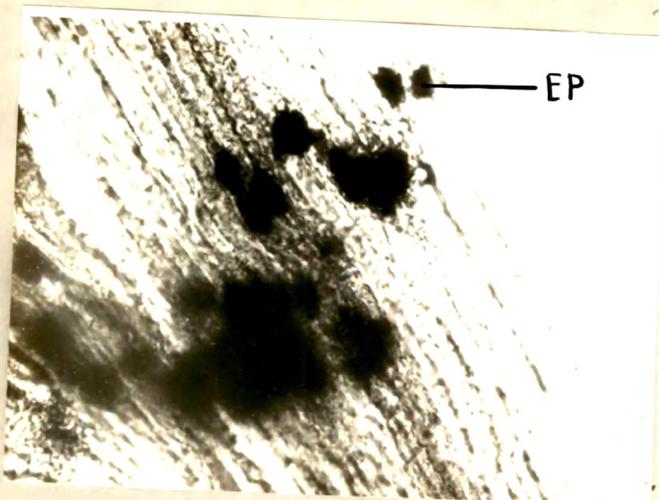


Fig.6

Figs.4,5 & 6
 Histochemical
 demonstration of
 Butyryl choline-
 sterase in the
 Diaphragm of Fox.
 Muscle sections
 (longitudinal)
 stained for the
 localization of
 BuChE exhibit
 the "en plaque"
 type of nerve-
 endings. Figs.
 4, 5 & 6 are for
 dorsal, lateral
 and ventral
 regions respec-
 tively. Nerve-
 endings are marked
 with 'EP'.
 All 128 X.