CHAPTER 8

ACID AND NEUTRAL ATPases IN SKELETAL MUSCLE

Studies on the different types of adenosinetriphosphatase (ATPase) activity in the various tissues in relation to the effects of Mg and Ca ions, pH, substrate concentration and their response to activators (dinitrophenol, DNP; 2,3, dimercapto-1-propanol, BAL; L-cysteine) and inhibitors (p-hydroxymercuribenzoate, PCMB; salyrganic acid) have been carried out in the recent years (Singer and Barron, 1944; Newman et al. (1950; Maengwyn-Davies et al. 1952; Chappell and Perry, 1955; Padykula and Herman, 1955; Freiman and Kaplan, 1960; Azzone et al. 1961; Hori and Chang, 1963). Investigations on the histochemical localization of ATPase in skeletal muscles have shown the occurrence of at least three different types of the enzyme designated as mitochondrial, myofibrillar and sarcotubular. Mitochondrial ATPase was demonstrated by Engel (1962) in the insect flight muscle using the Ca- ATPase method. Schulze and Wollenberger (1962, 1963) by light and electron microscopic examination of cardiac muscle revealed ATP cleavage in the mitochondrial cristae, in parts of the sarcoplasmic reticulum and on the sarcolemma. Barden and Lazarus (1964) studied the mitochondrial ATPase activity of normal mouse skeletal muscle and compared the results with those of dystrophic mice. They obtained strong activation of mitochondrial ATPase with Mg^{++}, upon addition of DNP to the medium. Sommer and Spach (1964) obtained ATPase activity in the myofibrils

and sarcoplasmic reticulum of the heart muscle of normal and abnormal dogs. Padykula and Gauthier (1963) have histochemically demonstrated mitochondrial, myofibrillar and sarcotubular ATPases in sections of the rat diaphragm. They observed that at pH 7.2 in the presence of cysteine the white fibres exhibited a higher sarcotubular ATPase activity than the red fibres. With DNP a higher mitochondrial ATPase activity in the red fibres than in the white fibres at the same pH, was obtained. The results obtained by Azzone et al. (1960) indicate that at acidic pH values, very little activation was induced by the addition of DNP in the mitochondria prepared from liver, heart and breast muscle of pigeon.

In the present chapter an attempt was made to elucidate the possibility of histochemical demonstration of both mitochondrial and sarcotubular ATPases in the red and white skeletal muscle fibres at different pH, in the absence of activators. Myofibrillar ATPase is already well established (Padykula and Herman, 1955) and hence not considered in the present investigation.

MATERIALS AND METHOD

The pigeon pectoralis muscle which is a mixed muscle of both red and white fibres and the fish (Labeo rohita) trunk muscle consisting of the red and white fibres separately in two regions, were chosen as the experimental material. The rat diaphragm (ventrolateral region) was also studied for comparison with these. Small pieces of the muscle were removed and dropped

directly into liquid oxygen at -182°C for 10 seconds. Cryostat sections were cut at 6 to 8 μ and affixed to the coverslips in the usual manner.

For the demonstration of mitochondrial and sarcotubular ATPases, sections were directly incubated without fixation according to the method of Wachstein and Meisel (1957). The incubation medium contained 5mg adenosinetriphosphate (ATP), 4 ml H_2O; 2% lead nitrate, 0.6 ml; 0.1 M magnesium sulphate, 1 ml; 0.2 M Tris-HCl buffer, 4 ml; and distilled water, 0.4 ml. Sections were incubated at 37°C for 1 to 2 hours for fish and pigeon muscles and 15 to 20 minutes for rat diaphragm. Sections of fish muscle were incubated at 37°C because at this temperature the required period of incubation was found to be less. After treatment of the sections with 1% yellow ammonium sulphide, the sections were mounted in glycerine jelly. The effect on mitochondrial and sarcotubular ATPases at different pH, ranging from 1.5 to 7.2 was studied. The pH of the incubation medium was always checked after incubation and was found to be less than 0.2 pH units from their initial values. Activators such as DNP (5×10^{-4} M) and cysteine (0.0025 M) were employed only at pH 7.2 according to the procedures described by Padykula and Gauthier (1963).

Control sections were simultaneously incubated in the media without ATP or in media containing equimolar amounts of β-glycerophosphate substituted for ATP at the acid range. Another set of control sections were heated in water at 70°C

for 3 hours prior to incubation.

RESULTS

Histochemical demonstration of ATPase activity in muscle sections was assayed with the incubation medium set at the following pH : 1.5, 2.5, 4.0, 5.6 and 7.2. The muscles of all three animals studied showed strongest sarcotubular ATPase activity at pH 2.5, and very slight at pH 7.2. These results were without benefit of activators to stimulate the reaction. It seems likely that traces of enzyme reaction representing mitochondrial ATPase were also discernible at the various pH employed.

At pH 1.5, sarcotubular ATPase activity was found to be completely absent even after prolonged incubation (Fig. 7). The dot like structures seen in Fig. 7 appear to represent ATPase activity in the mitochondria. At pH 2.5, a more intensive enzyme activity was demonstrable in locations corresponding to the sarcotubular system of white fibres of the pigeon, fish and rat skeletal muscles. The greater sarcotubular ATPase activity in the white fibres of the pigeon breast muscle is to be expected because the sarcotubular system is more elaborate in these than in the red fibres. The central part of the white fibre was stained darker than the peripheral part. ATPase activity was also seen to be located in sites which resembled the nuclei, present at the periphery of the red fibres and within the substance of the white fibres (Fig. 1 a & b). On staining with haematoxylin, it was confirmed

that these sites of ATPase activity are actually nuclei (Fig. 2). Novikoff et al. (1958) have reported the absence of nuclear staining for ATPase, and referred to the localization of ATPase in the nucleus as artifacts. Nevertheless, Sandler and Bourne (1962) obtained nuclear activity with the lead as well as the calcium methods, but only when magnesium nitrate and not magnesium sulphate was used as an ingredient of the incubation medium. They were able to produce or banish nuclear ATPase activity at will according to the amount of magnesium sulphate used. In view of the existing controversy with respect to this problem, it may be stated that in the present study the ATPase activity was clearly distinguished in the nuclei at pH 2.5.

In the fish muscle at pH 2.5 the white fibres showed intense enzyme activity in the sarcoplasmic reticulum (Fig. 3) which is in the form of a network in the central part of the fibre and traverses radially towards the periphery as described by Veratti (1902). In the red fibres too the enzyme activity was intense and mainly confined to the sarcoplasmic reticulum though dots similar to mitochondrial sites were also seen (Fig. 4).

With regard to the rat diaphragm, it was found that at pH 2.5 the distribution of enzyme activity also suggested localization chiefly in the sarcotubular system with the white fibres showing intense activity and the red very little (Fig. 5 a & b).

Incubations at pH 4.0 (Fig. 6 a & b) and 5.6 gave results similar to those obtained at pH 2.5 but the intensity

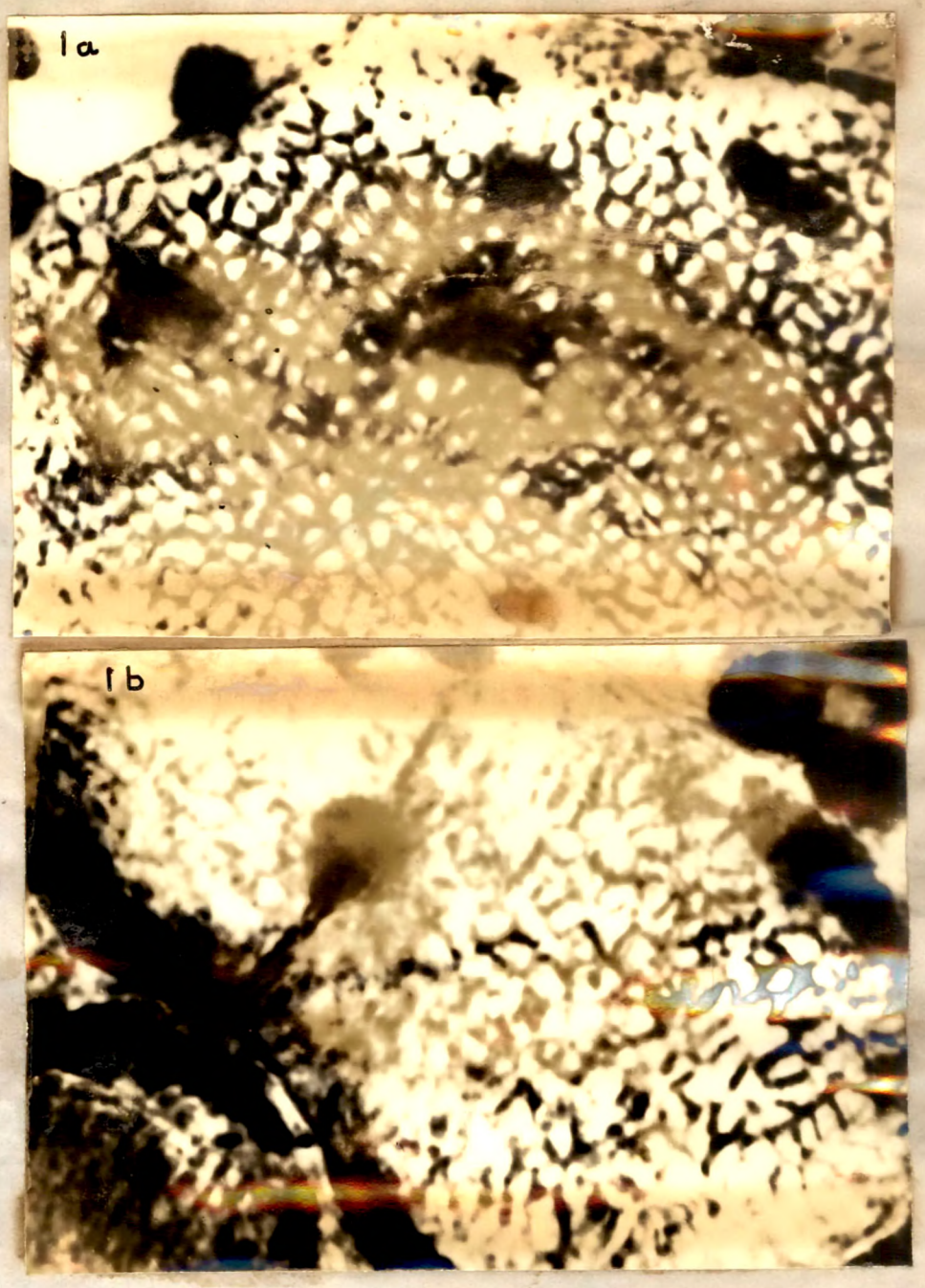


Fig. 1 a & b Transverse section of the pigeon pectoralis showing ATPase activity at pH 2.5. The sarcoplasmic reticulum which is more prominent in the white fibre (a) is well stained throughout the fibre. The nuclei in the white fibre are seen inside the fibre whereas in the red fibres (b) they are peripherally placed beneath the sarcolemma. Incubation time 2 hours. X 2520

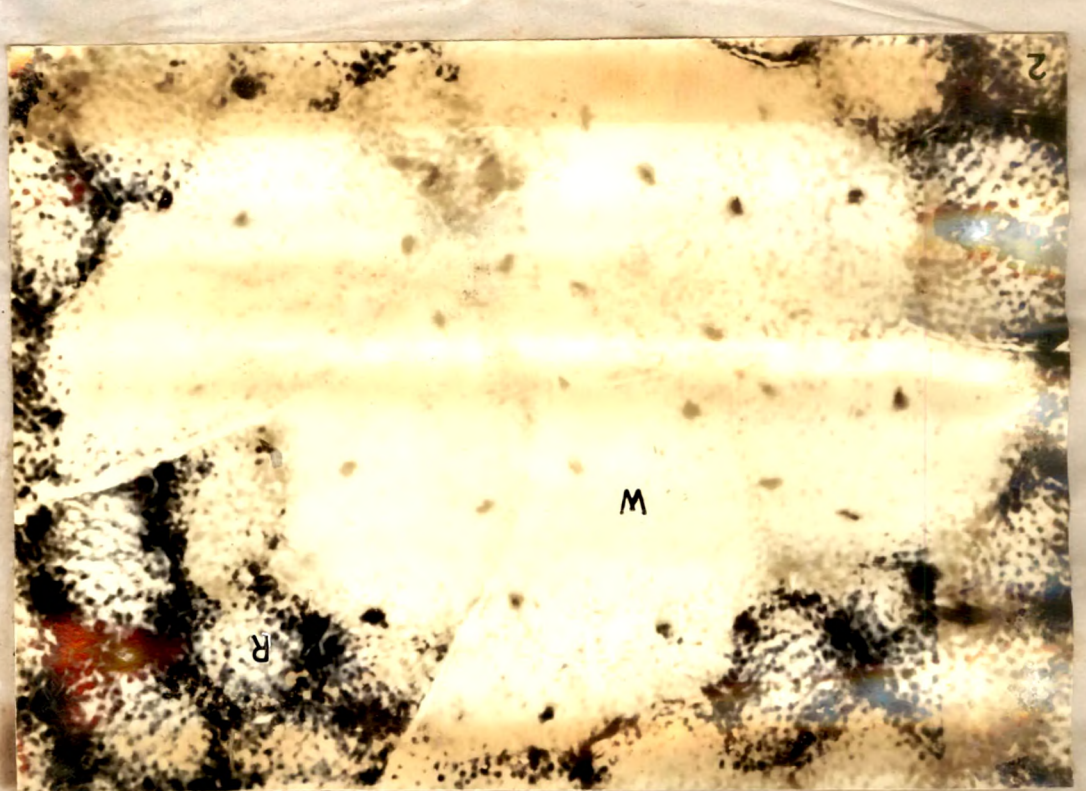
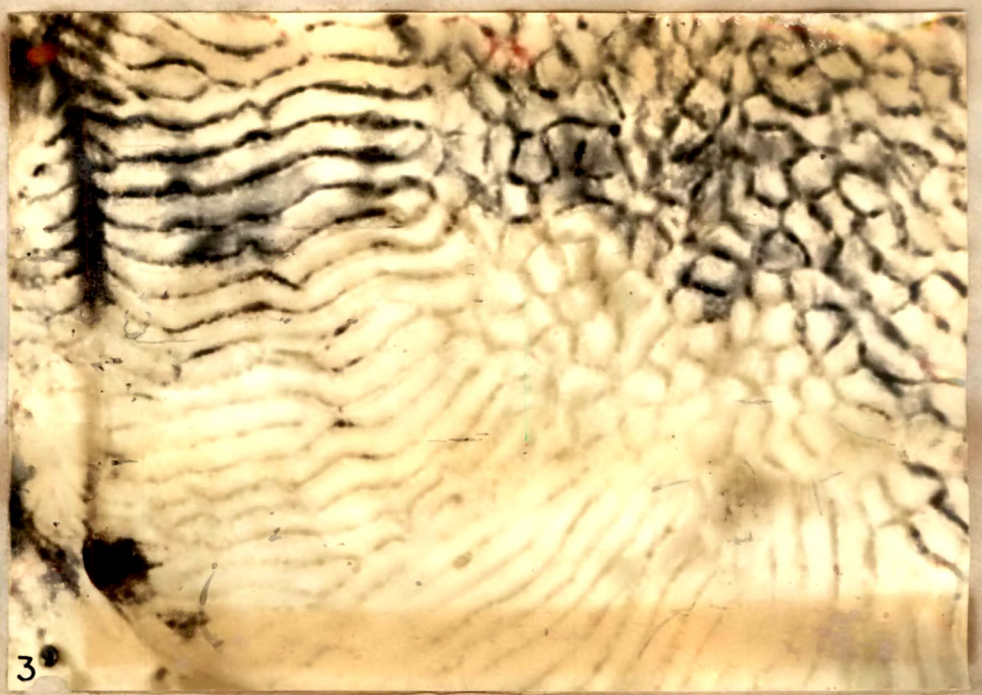


Fig. 2 Transverse section of the pigeon pectoralis stained with haematoxylin to show the location of the nuclei. Note the presence of nuclei within the substance of the white fibres (W) and in the red fibres (R) at the periphery. X 720



Figs. 3 & 4 Transverse sections passing through the white and red muscle regions respectively of the fish. ATPase activity at pH 2.5 is mainly localized in strands which correspond to the sarcoplasmic reticulum. Fig. 3 shows a single white fibre with a large area of the central sarcoplasmic core having a reticular network, extensions from which are seen to traverse the fibre radially towards the periphery. Incubation time 1 hour. Magnification in both cases X 1800

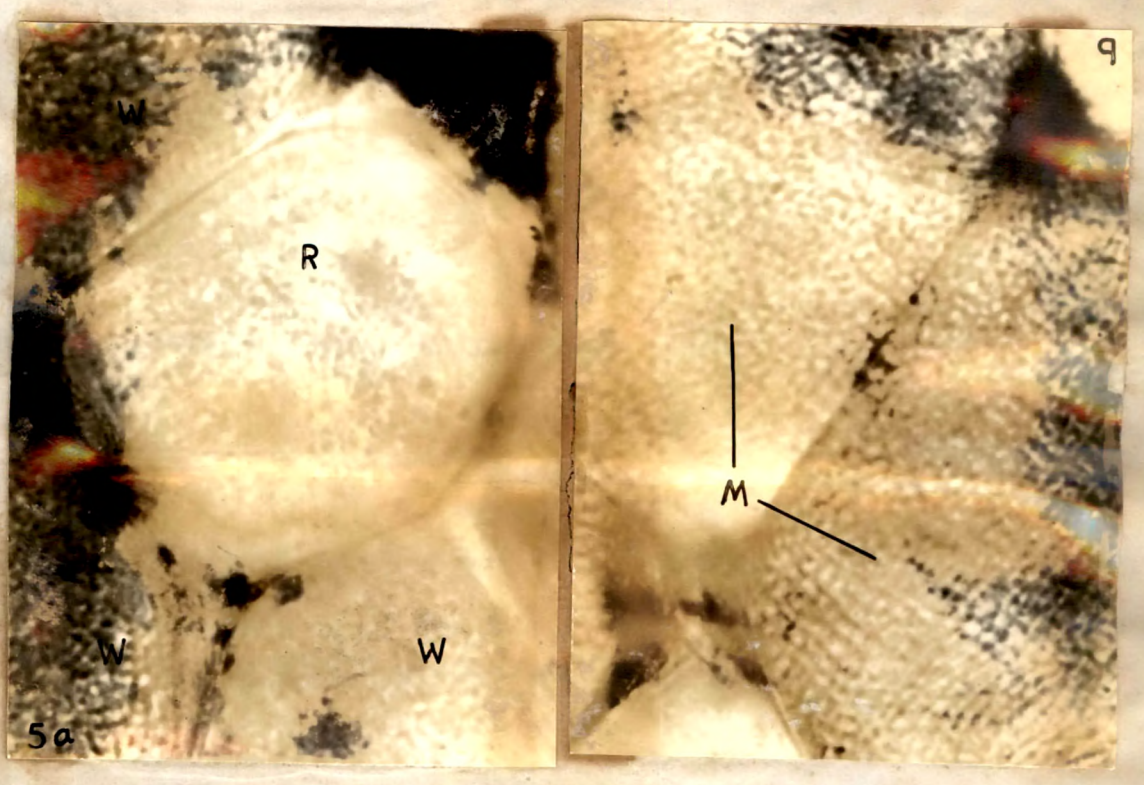


Fig. 5 a & b Transverse section of the rat diaphragm showing ATPase activity at pH 2.5. The reticular network of strands within the broad white fibres (W) appears to represent the sarcoplasmic reticulum. Some of the reactive dots seen within the fibres seem to indicate the mitochondrial activity as well. The red fibre (R) is faintly reactive as compared to the broad white ones. Incubation time 15 to 20 minutes. X 1800

Fig. 6 a & b Transverse section of the pigeon pectoralis demonstrating ATPase activity at pH 4.0. Staining is seen in the sarcoplasmic reticulum which is more elaborate in the white fibres (a). The pattern of staining seen in the red (b) as well as white (a) fibres indicates that some activity may also be present in the mitochondria. Note that the staining of nuclei is not evident at this pH. Incubation time 3 hours. X 1800

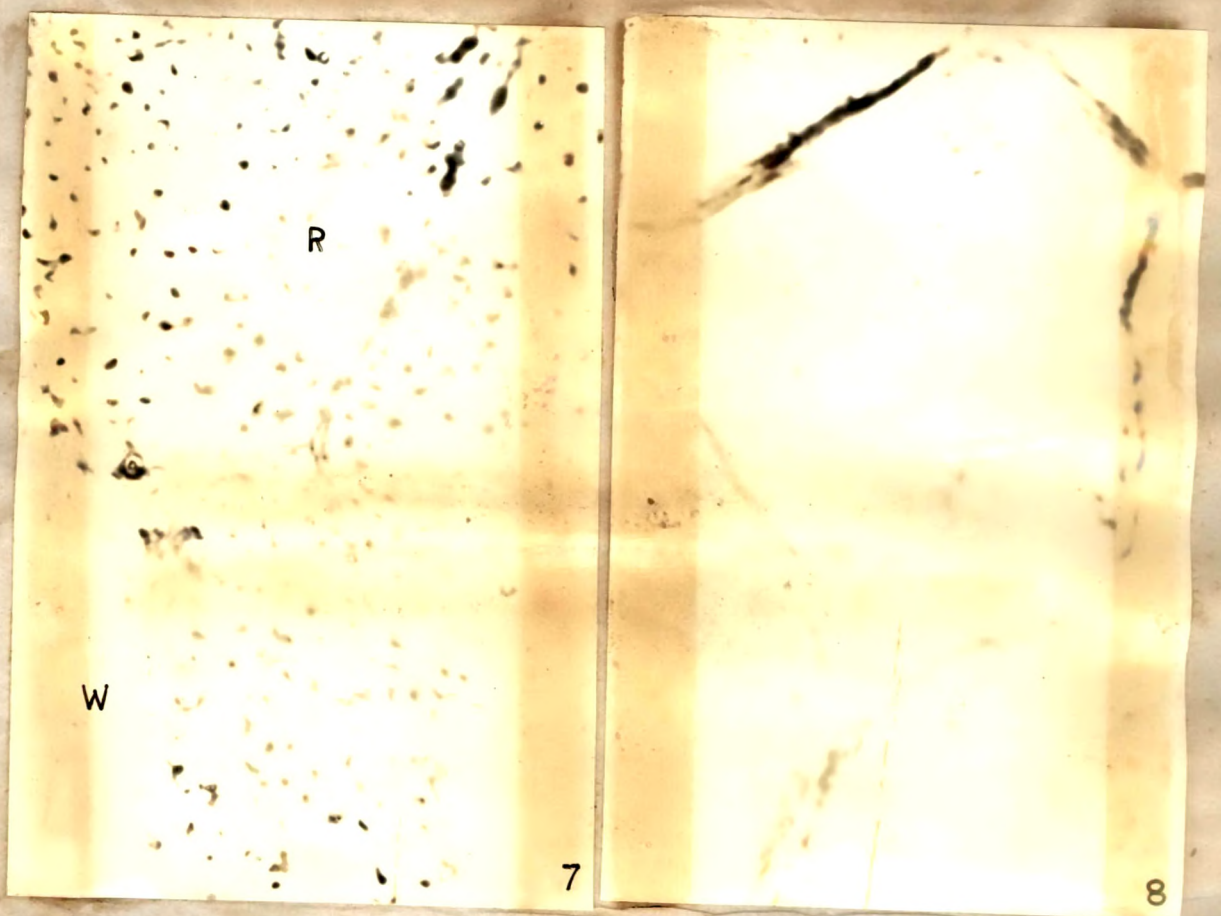


Fig. 7 Transverse section of the pigeon pectoralis incubated at pH 1.5 for the demonstration of ATPase activity. Sarcotubular ATPase activity is found to be totally absent at this pH in both the white (W) and the red (R) fibres. Slight reaction is also seen at sites corresponding to mitochondria. Incubation time 3 hours. X 1800

Fig. 8 Control section of the pigeon pectoralis heated for 3 hours prior to incubation at pH 2.5. Hardly any reaction is seen in the sarcoplasmic reticulum as well as mitochondria. Incubation time 3 hours. X 1800

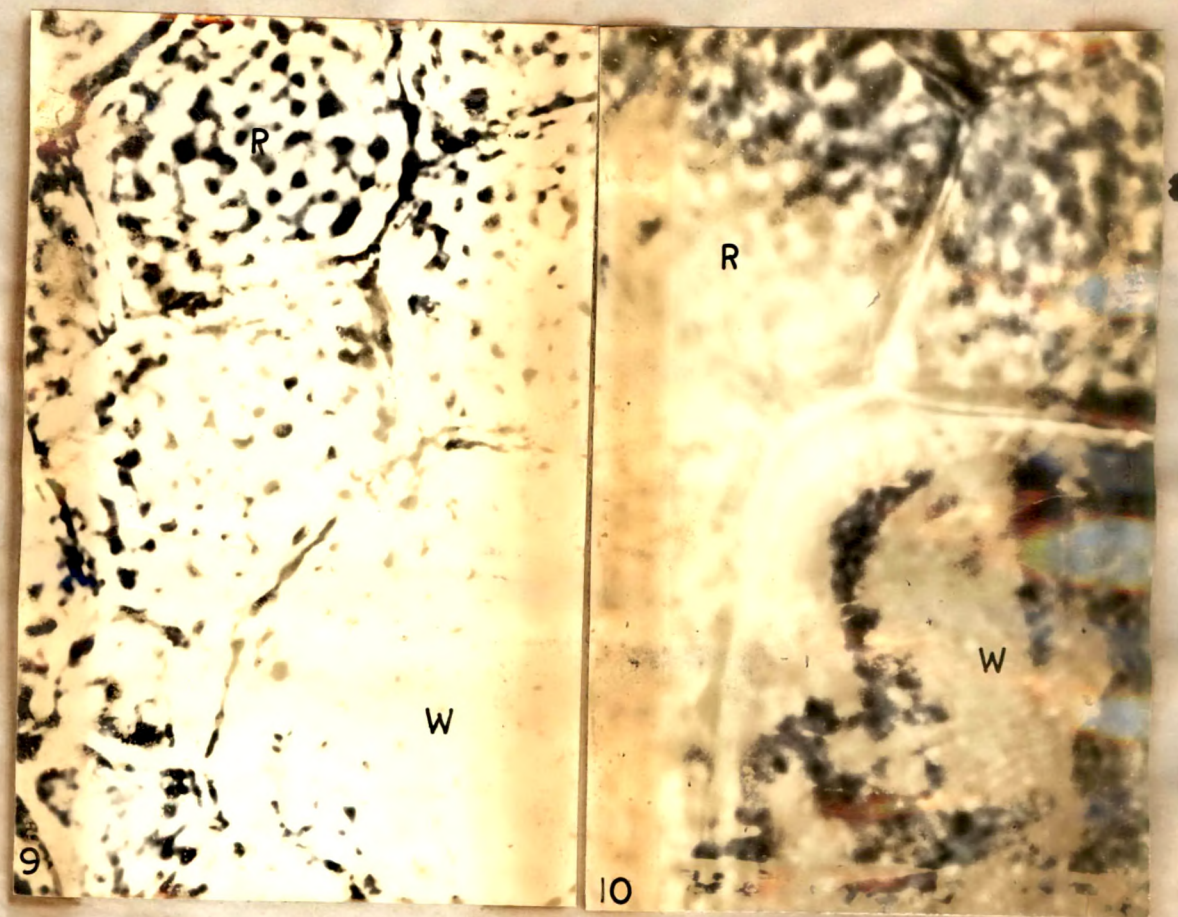


Fig. 9 Transverse section of the pigeon pectoralis showing ATPase activity at pH 7.2 after incorporating DNP in the incubation medium. ATPase activity is stimulated at sites corresponding to the mitochondria which are larger and more numerous in the red (R) fibres. Incubation time 3.5 hours. The section was fixed in calcium formol prior to incubation. X 1600

Fig. 10 Transverse section of the pigeon pectoralis showing ATPase activity at pH 7.2 with cysteine added to the medium. A prominent reticulum is seen in the white fibre (W). In addition, granular deposits corresponding to mitochondria are seen in both the red (R) and white (W) fibres. Incubation time 4 hours. X 1800

of the reaction was highest at pH 2.5. At pH 7.2 it was observed that though there was an overall diminution in the intensity of the reaction for sarcotubular and mitochondrial ATPases even after prolonged incubation, the enzyme activity in the mitochondria of the red fibres was relatively greater than in the white fibres. At this pH, mitochondrial ATPase activity increased considerably when 5×10^{-4} M DNP was incorporated into the medium (Fig. 9). When cysteine (0.0025 M) was added to the incubation medium at pH 7.2, in addition to mitochondrial activity, sarcotubular activity was also seen (Fig. 10), as was observed by Padykula and Gauthier (1963) in the fibres of the rat diaphragm.

Control sections incubated in a medium lacking ATP or which were heated in water for 3 hours prior to incubation, showed a negative staining reaction (Fig. 8).

DISCUSSION

From the results obtained it is evident that a sarcotubular ATPase can be demonstrated at acidic pH values as low as 2.5. Intense sarcotubular ATPase activity is seen in the quick contracting white fibres in which the reticulum is considerably more developed than in the red fibres. Peachey and Porter (1959) suggested that the speed of contraction may be related to the degree of development of the reticulum. Studies on the swim bladder muscle of the toadfish (Fawcett and Revel, 1961), cricothyroid muscles of the bat (Revel, 1962), the synchronous flight muscles of the dragonfly (Smith, 1961) and the extrinsic

eye muscles of Fundulus (Reger, 1961), all of which are fast muscles have revealed a similar extensively developed reticulum. The sarcoplasmic reticulum is also found to be more pronounced in cases where the muscle is designed to return to the relaxed state in a few milliseconds (Skoglund, 1961), and is far less prominent in those muscles that relax slowly.

Muscatello et al. (1962) from their studies on frog skeletal muscle homogenates have reported that fragments originating from the sarcotubular system are associated with the production of the relaxing factor, and that the sarcotubular system is the only structure of the muscle cell where the relaxing factor can be synthesized. Furthermore, they have inferred that the synthesis of the relaxing factor takes place through the operation of the sarcotubular ATPase, which supplies the energy required for the synthesis of the factor. This sarcotubular ATPase may, therefore, be functionally related to the mechanism of muscle relaxation.

It has been suggested that the sarcoplasmic reticulum plays an important role in spreading the impulse from the sarcolemma to the contractile elements in the interior of the muscle fibres (Porter and Palade, 1957; Huxley and Taylor, 1958; Ruska et al. 1958; Peachey and Porter, 1959 and Huxley, 1959, 1964). The role of the sarcoplasmic reticulum with regard to the synthesis of the relaxing factor has been mentioned earlier. This lends further support for the participation of the sarcoplasmic reticulum in the mechanism linking excitation

and contraction. It has been found that there is on electrical stimulation, a remarkable increase in sarcotubular ATPase (pH 2.5) activity in the gastrocnemius muscle of the frog (Chapter 9).

The appearance of lactic acid as a result of muscular exercise was first demonstrated by Fletcher and Hopkins (1907), and later Meyerhof (1920) showed that the source of this lactic acid was glycogen stored in the muscle. It has been suggested in salmonoid fishes, that the rate of blood flow through the heart is substantially reduced after muscular exercise (Black et al. 1955, 1956, 1957a, b, c, 1959 and 1960). This slow rate of circulation may also contribute to the delay in the removal of lactate from muscles and blood (Black, 1957c). According to Amlacher (1961) there exists a close relationship between the pH value and the electrical resistance of the muscle. The latter decreases with the fall of hydrogen ion concentration, caused by the higher permeability of the muscle tissue to free ions. From this stage on, the process of glycolysis which increases enormously following stimulation (Cori, 1956), slows down until all the glycogen is consumed or till the glycolytic enzyme system becomes inactivated at pH 5.6 (Bate-Smith, 1948). As long as anaerobic glycolysis continues, 1.5 moles of ATP are formed from ADP for every mole of lactic acid arising from glycogen (Bate-Smith and Bendall, 1949). The presence of ATP and magnesium, in addition to the relaxing factor, is essential for bringing about relaxation of the muscle (Bendall, 1954). Perhaps, the

high concentration of the sarcotubular ATPase activity observed at acid pH in the present study, may be considered an adaptation for the synthesis of the relaxing factor. When pH is thus lowered, it should be of advantage to have the sarcotubular ATPase adapted to a lower pH. Under the histochemical conditions of the present investigation it also appears that ATP hydrolysis may be facilitated by the low pH. However, it is unlikely that *in vivo* the pH conditions in these fibres would ever reach such low levels.

Andersson-Cedergren and Muscatello (1963) found the UDPG glycogen synthetase activity to be localized in the sarcotubular fraction and considered this as conclusive evidence in the participation of the sarcotubular system in the synthesis of glycogen. The high ATPase activity at pH 2.5 and the correspondingly low activity at pH 7.2 in the sarcoplasmic reticulum of the white fibres may be related to the metabolic requirements of the fibre at the lower pH range. The red fibres on the other hand, are adapted for an aerobic metabolism and can readily metabolize fat (George and Talesara, 1962). In these fibres in which suitable physiological conditions such as myoglobin for copious oxygen supply and numerous mitochondria containing high concentrations of oxidative enzymes exist, the occurrence of a mitochondrial ATPase acting in an alkaline pH is of distinct advantage. That an alkaline pH is better suited for oxidative metabolism has been shown by Nanni *et al.* (1963) using isolated

mitochondria *in vitro*. They demonstrated that oxidative metabolism and QO_2 value increased gradually at alkaline pH from 6.0 to 8.0 and advanced a hypothesis that modifications of pH in the cell, control a mechanism for autoregulation of mitochondrial functions.

The sarcotubular ATPase activity obtained in the present study was found to be localized in a network in between the myofibrils in the acidic pH range (2.5 to 5.6) in the presence of magnesium ions. The addition of cysteine to the incubation medium found necessary by Padykula and Gauthier (1963) for the demonstration of sarcotubular ATPase at pH 7.2 was not found necessary for demonstrating ATPase activity at the acidic pH. This indicates that sarcotubular ATPase acting in the acidic range is not sulphydryl dependent and is different from the sulphydryl dependent ATPase of Padykula and Gauthier acting at pH 7.2.

In the light of the discussion above, it appears probable that in skeletal muscle, there are two types of sarcotubular ATPase with regard to pH optima -- an acid ATPase which does not require any sulphydryl compound for its demonstration and a neutral ATPase which shows a distinct reaction only on the addition of a sulphydryl compound, both however, requiring the presence of magnesium ions. It may therefore be concluded that pH 2.5 is the optimum for the acid ATPase and 7.2 for the neutral ATPase.