CHAPTER 1

HISTOCHEMICAL CHANGES IN GLYCOGEN AND PHOSPHORYLASE IN THE PIGEON PECTORALIS DURING DISUSE ATROPHY

It has been shown earlier that when the pigeon breast muscle is subjected to disuse atrophy, several physiological changes take place in the muscle and blood (George and Vallyathan, 1962). These changes include striking differences particularly during the initial period of atrophy, in the fat and water contents and in the lipase activity, thereby indicating a severe disturbance in the lipid metabolism of the muscle. The observation of a sharp increase in the lipid content 24 hours after subjecting the muscle to disuse, is tempting to suggest that fat is being formed in the muscle from other metabolites. It is already known that there is destruction of muscle protein during muscular atrophy (Fisher and Ramsay, 1946). Hines and Knowlton (1935) and Lazere et al.(1943) working with mammalian material showed that there is a decrease in glycogen concentration following denervation of the muscle. However, changes in the activity of muscle phosphorylase observed by many investigators do not parallel closely the concomitant changes in glycogen concentration. Arson and Volk (1957) reported slight decrease in phosphorylase activity during the first week of atrophy followed by a rapid decrease later. Humoller et al. (1951) recorded steady increase in phosphorylase activity beginning immediately after denervation of the muscle .

Recently Huls and Leonard (1961) have observed a slight increase in phosphorylase activity 5 days after denervation and then a decrease reaching the lowest level by 20th day. In contrast to these findings Matsui and Kuriaki (1959) observed increasing phosphorylase activity upto 20 days after denervation of the muscle.

Since the available data on the glycogen and phosphorylase levels in the muscle under disuse are conflicting, a detailed histochemical study of glycogen and phosphorylase in the pigeon pectoralis muscle was carried out in order to obtain a clearer picture of their localization and also to correlate the finding with quantitative data. Pigeon pectoralis was selected since it is known that it is a metabolically active muscle consisting of two types of fibres (red and white) specialized for aerobic and anaerobic metabolism respectively.

Material and Methods

Adult healthy pigeons weighing 290-320 gm maintained on a standard diet of mixed grains were used. A plaster cast was applied at the shoulder joint after restraining the wings in a back to back position as described by George and Vallyathan (1962). At the end of the required period the pigeon was killed by decapitation and pieces of the breast muscle were cut out for investigation.

Glycogen: Pieces of muscle were dropped in cold (-4°C) alcoholic picroformol and fixed for 24 hours. After washing in alcohol and further clearing, they were embedded in paraffin wax.

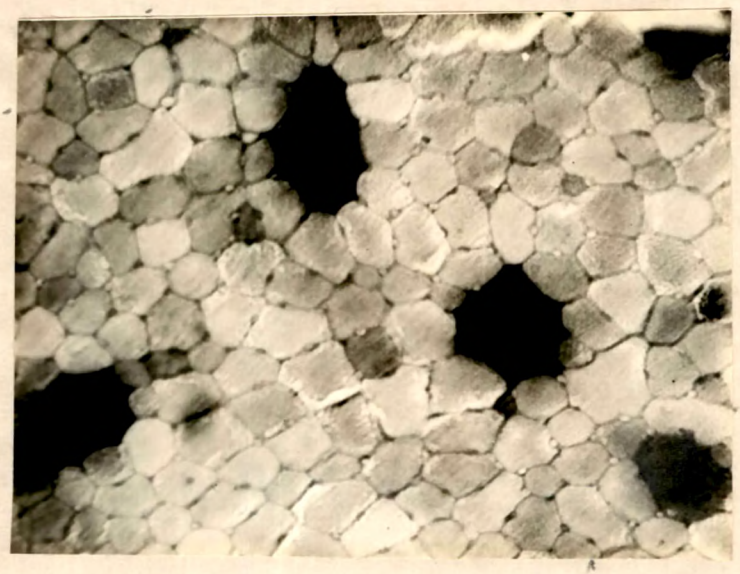
Sections of the embedded tissue were cut (5-8μ) and stained for glycogen employing the periodic acid Schiff (PAS) technique (Pearse, 1960). Sections treated with saliva for 30 minutes at 37°C served as control.

Phosphorylase: Histochemical demonstration of phosphorylase activity was carried out according to the method of Eränkö and Palkama (1962) using glucose-1-phosphate as the substrate. A piece of breast muscle was quickly excised and frozen on the stage of a freezing microtome. Sections 15 to 20 μ thick were cut and dropped directly into the incubation medium and incubated at 30°C for 15 minutes. After incubation the sections were rinsed in 40% alcohol, brought to water and stained with Gram's iodine solution for 1 to 3 minutes. Unincubated sections treated with iodine and mounted in glycerine jelly formed the control.

Results

The normal pigeon muscle is shown to illustrate the distinct differences between the two type of fibres. The broad white fibres show high concentrations of phosphorylase and glycogen, but the narrow red fibres contain very little glycogen and phosphorylase activity(Figs. 1 and 2). In atrophy distinct histochemical differences between the two types of fibres appeared only after 7 days.

Glycogen: In 7 day atrophy the majority of the red fibres were found to be more PAS positive, and white fibres less so, than their normal counterparts (Fig. 3). The red fibres stained



Magnification in all cases 400X. Incubation time for phos phorylase in all cases 15 minutes. Fig. 1. Normal pigeon breast muscle stained for glycogen using the periodic acid Schiff technique (experimental control). Note the higher concentration of glycogen in the broad white fibres.

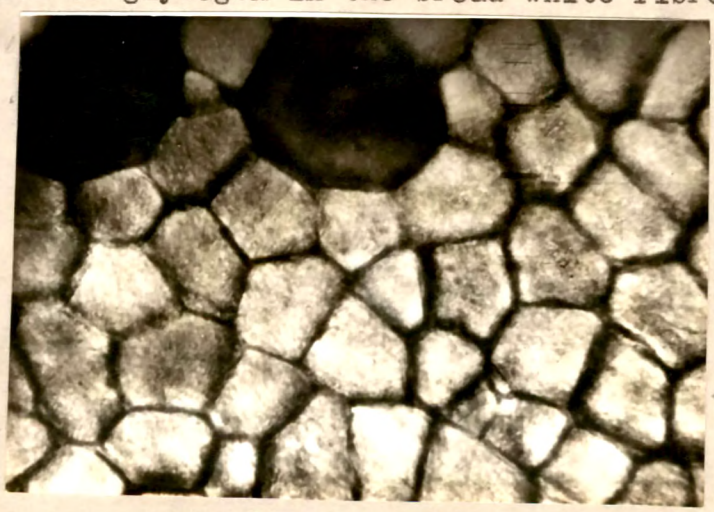


Fig. 2. Normal pigeon breast muscle stained for the demon - stration of phosphorylase with Eränkö and Palkama technique (experimental control). Darkly stained (blue) broad white fibres show greater enzyme ativity than the narrow fibres showing a negative reaction.

variably. Later by the 14th and 21st days of atrophy, these changes became sharper and the concentration of glycogen appeared to be higher in the red fibres (Figs. 5 and 7). By the 30th day of atrophy, however the white fibres were regaining their glycogen load while the glycogen in the majority of the red fibres was found to be depleted (Fig. 11).

Phosphorylase: Phosphorylase activity in the control sections revealed by the reaction of the iodine with the freshly formed glycogen, appeared dark blue in the white fibres, but slightly violet (magenta) in the red fibres (Fig. 2). In 7 days atrophy, the red fibres showed a much deeper magenta colour, whereas the white fibres were blue (Fig. 4). Among the red fibres some were found to stain blue, some violet, and others not at all. By the 14th day, the majority of the white fibres lost their enzyme activity but the red ones acquired a higher and uniform level of enzyme activity (Fig. 6). By the end of 21 days the white fibres regained their normal enzyme level but the red fibres showed a lower level of activity (Fig. 8). In 30 as well as 60 day atrophy, the white fibres were found to have as high enzyme activity as the controls (Figs. 10 and 12). The majority of red fibres by this time showed a uniform staining higher than in the controls, but a few of the white as well as the red fibres appeared to be devoid of the enzyme. Such fibres were also present in sections stained for glycogen. The white fibres lacking in enzyme activity and glycogen, with continued atrophy, were found to be gradually accumulating fat in them and replaced by

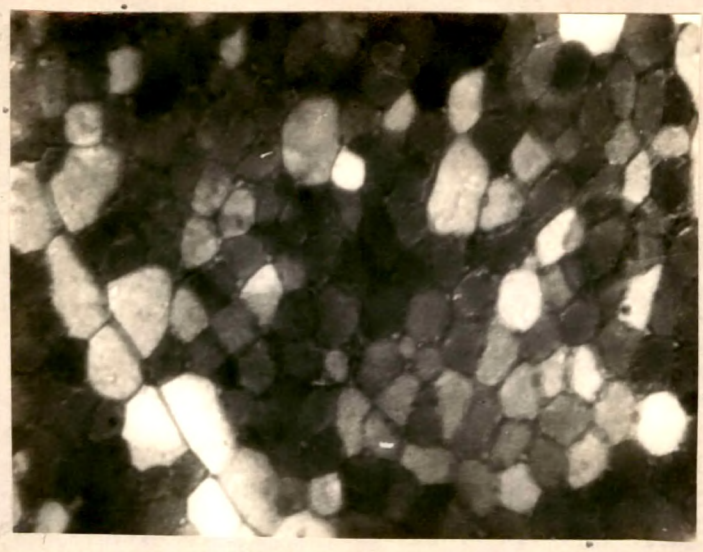


Fig. 3. Glycogen in 7 days atrophy. Broad fibres contain little glycogen whereas the narrow ones show higher concentration compared to the normal muscle.

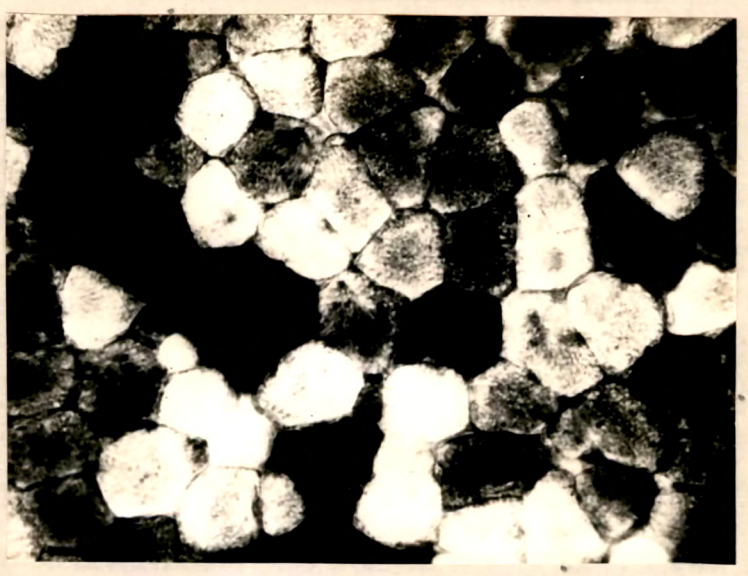


Fig. 4. Phosphorylase after 7 days atrophy. Note some of the narrow fibres show enzyme activity (stained violet).

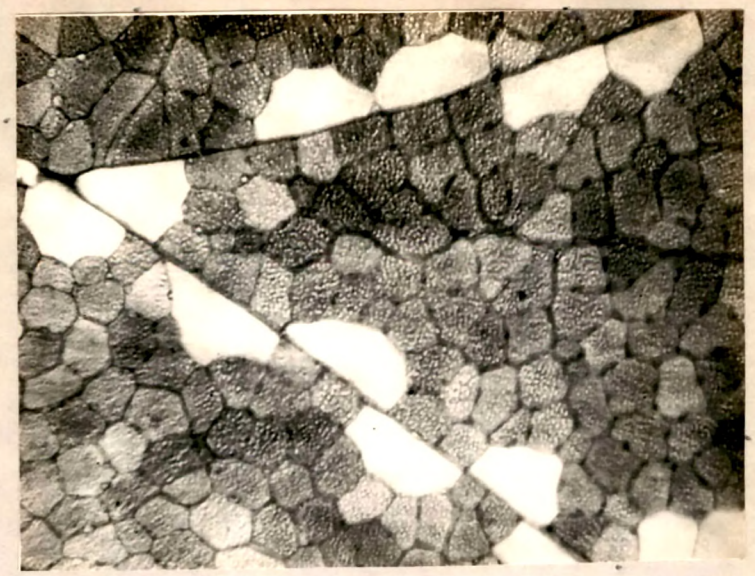


Fig. 5. Glycogen after 14 days atrophy. The broad white fibres are completely periodic acid Schiff negative and the narrow ones show a uniform staining.

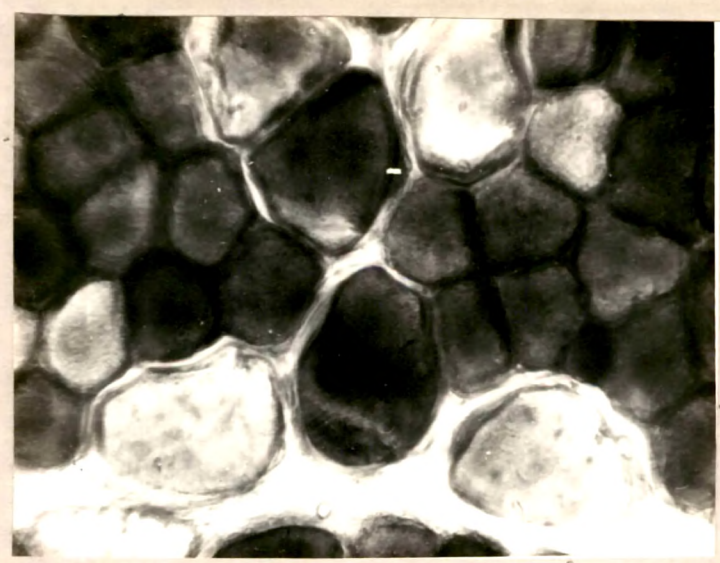


Fig.6. Phosphorylase after 14 days atrophy. Some broad fibres show no enzyme activity while others do. Majority of the narrow fibres show a uniform staining reaction .

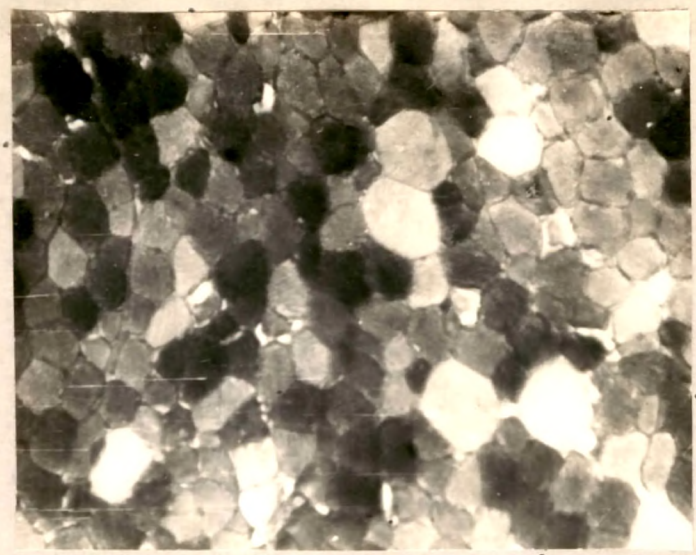


Fig. 7. Glycogen after 21 days atrophy. Broad fibres are periodic acid Schiff negative. Few narrow fibres show increased staining while a few are periodic acid Schiff negative.

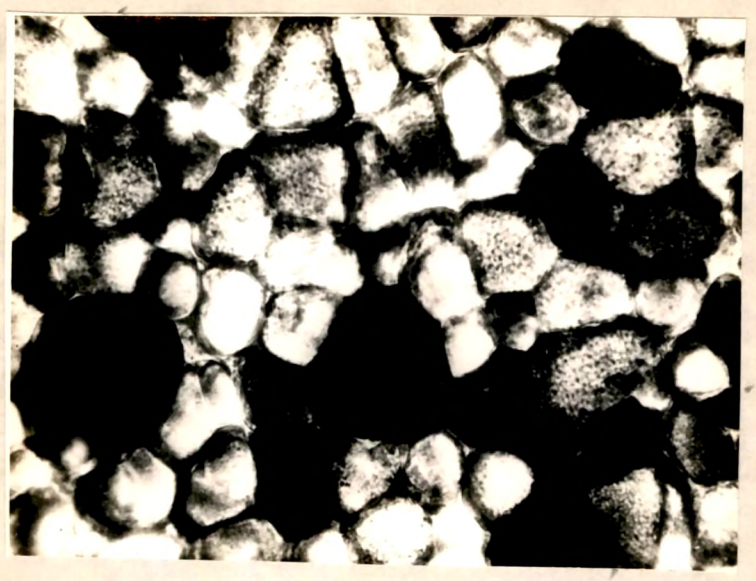


Fig. 8. Phosphorylase after 21 days atrophy. Broad fibres darkly stained and the narrow ones lightly.

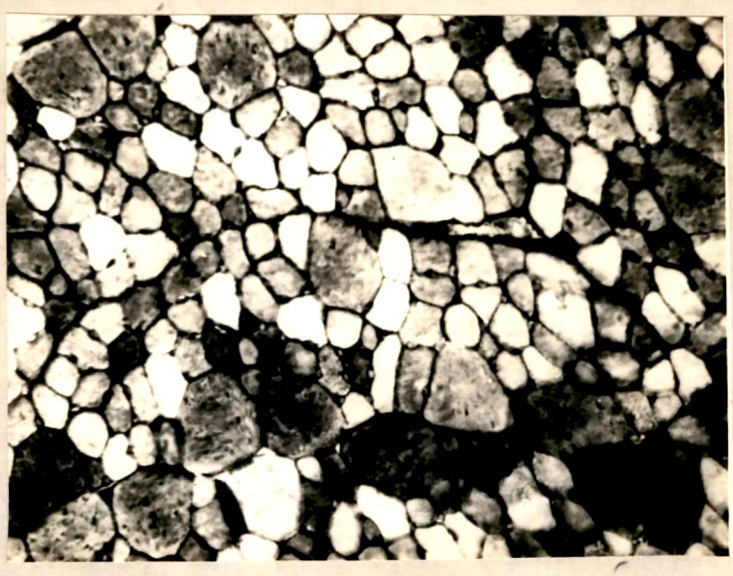


Fig. 9. Glycogen after 30 days atrophy. Note the reappearance of the periodic acid Schiff positive staining in the broad fibres. Narrow fibres present different grades of staining reaction.

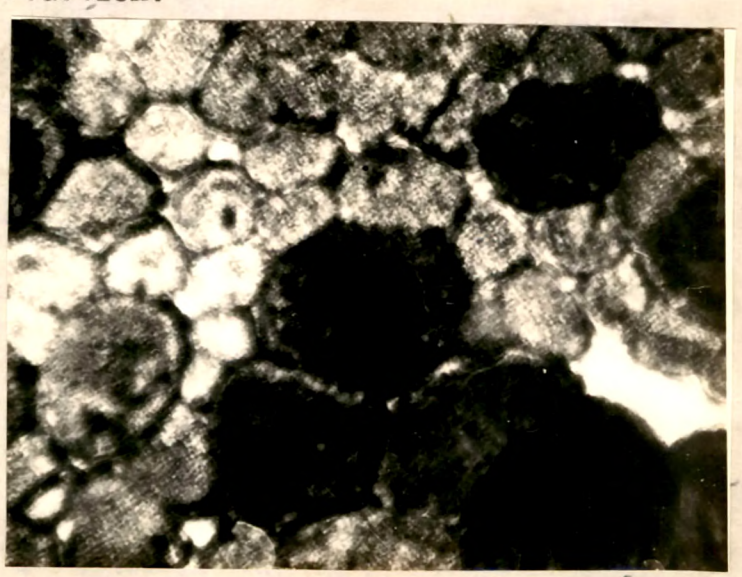


Fig. 10. Phosphorylase after 30 days of atrophy. Varying enzyme concentration in both the types of fibres.

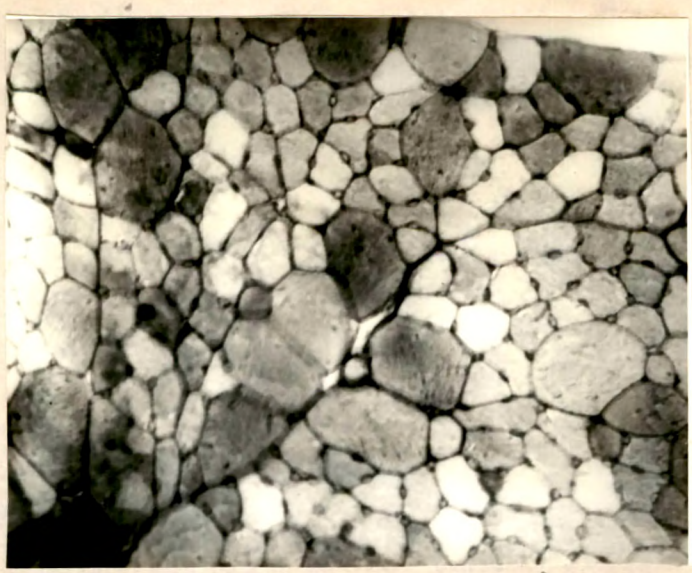


Fig. 11. Glycogen after 60 days atrophy. Broad fibres periodic Schiff positive but less intense reaction than normal. Narrow ones uniformly stained.

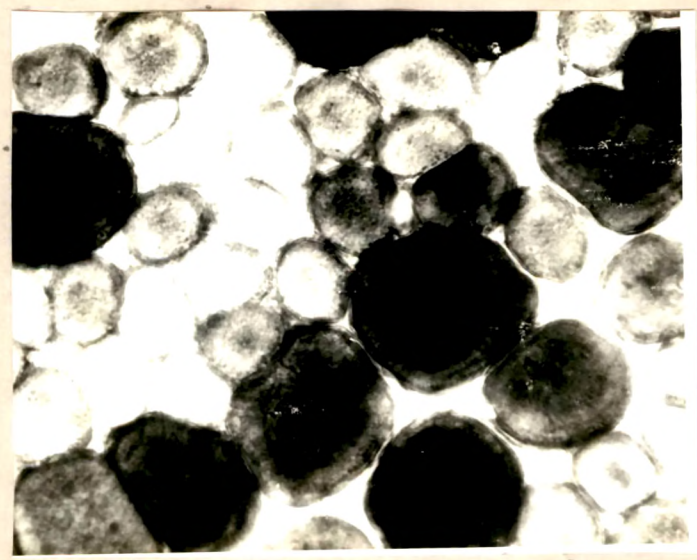


Fig. 12. Phosphorylase after 60 days atrophy. Broad fibres and some of the narrow ones stained dark while some narrow ones show no enzyme activity.

connective tissue. Such fibres were reduced in size or found to be oblong or oval in shape. Finally these white fibres disintegrated completely. On the other hand, very few of the red fibres changed in this way. However, in certain regions an entire fasciculus containing both types of fibres was found to be replaced by fat and connective tissue, leaving a few red fibres intact. There was no phosphorylase activity in the connective tissue.

Discussion

The importance of glycogen as an energy source and the role of enzyme phosphorylase in the initial reversible reaction effecting the synthesis and breakdown of the 1,4-glycosidic linkages is well recognized (Stetten and Stetten, 1960). Hence changes in phosphorylase activity can be considered to reflect changes in glycogen metabolism. In the pigeon breast muscle higher phosphorylase activity was demonstrated in the broad white fibres than in the narrow red fibres histochemically by Dubowitz and Pearse(1960) and quantitatively by Vallyathan and George (1963).

Polysaccharides newly synthesised enzymically from glucose-1-phosphate in histochemical preparations have been shown to differ in their physicochemical nature from the native glycogen. Polysaccarides formed by amylophosphorylase activity stains blue with iodine, while that formed by combined amylophosphorylase and the branching enzyme stains violet or purple (Sasako and Takeuchi, 1963). In the present study on the pigeon

breast muscle, it was found that the white fibres stained dark blue and the red ones slightly purple in colour. During atrophy, however, the red fibres showed a more intense purple staining. Sasako and Takeuchi (1963) observed in the human gluteus muscle that the narrower red fibres were stained blue with iodine while the broad white ones purple. The difference observed in the staining reaction between the human gluteus and pigeon breast muscle fibres cannot be explained without comparative studies.

From the changes in the glycogen and phosphorylase during atrophy, it is clear that there is drastic change in the metabolism of the red and white fibres, which have been shown to be adapted for aerobic and anaerobic metabolism respectively in the normal bird (George and Talesara, 1962). However, the increase in enzyme activity in the red fibres during the course of atrophy presented a composite picture of amylophosphorylase and other branching enzyme activity.

The quantitative data on the whole muscle show that after an initial decrease there is an increase in glycogen and phosphorylase levels during disuse atrophy. Leonard (1957) and Huls and Leonard (1957) observed that muscle phosphorylase activity is reduced to a minimum after 20 days in dystrophic and denervated skeletal muscle, although they (1961) also reported a slight increase in enzyme activity after 5 days of denervation. In a quantitative study of phosphorylase in the pigeon breast muscle (Vallyathan, 1964) the enzyme level was found to decrease gradually to a minimum by the 7th day.

Thereafter, by the end of 60 days it had risen considerably, though not to the control level.

Following denervation, the muscle glycogen in rat has been shown to decrease more rapidly than the tissue mass (Lazere et al., 1943). Vallyathan (1964) also found a decrease in glycogen during disuse atrophy. The increase in glycogen in the narrow red fibres seen in the present study after the 7th day, corresponds to the higher values obtained by Vallyathan (1964) during the 14th and 21st days of atrophy.

In studies reported earlier (George and Vallyathan, 1962), it was suggested that there is a disturbance in the lipid metabolism of the muscle during the earlier days of atrophy and that the increase in muscle fat at the onset of atrophy may be possibly due to a de novo synthesis of fat from other metabolites which are being transformed at a rapid rate. The histochemical findings reported show that there are certain striking changes in the pattern of metabolism in the two types of fibres. The broad white fibres adapted for glycolytic metabolism underwent degeneration much more quickly than the red ones and showed considerable reduction in glycogen and phosphorylase. On the other hand, the red fibres, adapted for an oxidative metabolism, accumulate phosphorylase and glycogen during the course of atrophy.

If it is assumed that the rise in glycogen level in the red fibres is to resist the initial effects of atrophy, the question arises why there was no such increase in the white

fibres, which are specially adapted for glycolytic metabolism. George and Vallyathan (1962) have pointed out that there was no complete inactivation of the muscle when the wings were subjected to plaster cast. At all stages of disuse, a shivering movement in the wings could be seen. Therefore one possibility is that in the white fibres the glycolytic process is so fast and sustained that there is a continuous drain of glycogen. Another possibility is that in these fibres the amount of intracellular fat is so little that there is no possibility of utilizing fat, or glycogen build up from fat. On the other hand, in the red fibres a high glycogen level could be built up from the dense intracellular fat reserves. This could be achieved by increased lipolysis of fat to fatty acids and glycerol, and the conversion of the latter to glucose and finally to glycogen. The concentration of potassium observed during atrophy (Chapter 4) further give support to such a hypothesis, since it is known that an increase in potassium is parallel to the increase of glycogen (Fenn, 1938). Fatty acids may be oxidised for energy by increased oxidation in the muscle. That lipase activity is increased in muscle during this period has already been shown (George and Vallyathan, 1962). It should therefore be of interest to know whether the oxidative activity is also increased during this period. But a study in this regard has shown that the muscle <u>in vitro</u> is capable of oxidising fatty acids during this period (Chapter 5).