CHAPTER 7

EFFECT OF DENERVATION ON GLYCOGEN, PHOSPHORYLASE, SUCCINIC DEHYDROGENASE AND LIPASE LEVELS IN THE PIGEON BREAST MUSCLE

The importance of glycogen as an energy fuel for muscle and the role of the enzyme phosphorylase is fairly well known. From studies conducted on the pigeon breast muscle it is well established that during sustained muscular activity fat is utilized as the main energy fuel (George and Berger, 1965). It is also shown from <u>in vitro</u> experiments that the enzyme lipase is essential for the utilization of fat (George, 1964). It is also well known that the pigeon breast muscle consists of two types of fibres that differ strikingly in size, metabolic load and enzyme concentrations. The white glycogen-loaded, phosphorylase rich fibres are adapted for glycolytic metabolism and the red fat-loaded, fibres rich in oxidative enzymes and lipase for oxidative metabolism (George and Naik, 1958; George and Talesara, 1962;a&b ; Vallyathan and George, 1963).

The earlier histochemical investigations reported in chapters 1, 2, 3, 4 and 6 showed a number of biochemical changes in the energy metabolism of the two types of muscle fibres during induced muscular atrophy. These investigations have helped in defining the characteristic differences between the energy metabolism of the red and white types of fibres. From the correlation of the results with the physiological data reported in Chapter 5 and from the earlier investigations from this laboratory (George and Vallyathan, 1962; Vallyathan, 1963) it could be concluded that with the onset of atrophy there is a transition from an oxidative to a glycolytic pattern of metabolism in many of the red fibres and from glycolytic to oxidative in many of the white fibres. These studies have also shown a defect in the lipid metabolism of the muscle. Histochemically it has been impossible to delineate any of these fundamental mechanisms involved in this metabolic shift as a result of the limitations of the techniques employed.

The present biochemical investigation was therefore undertaken to confirm the earlier histochemical investigations and also to obtain a better understanding of the precise nature of the biochemical events taking place in the muscle during atrophy.

Material and Methods

Adult healthy pigeons, weighing 300-320 gm. were anaesthetized with ether. After making an incision at the base of the neck the brachial plexus of the right side was transected by removing 2mm. of the plexus at the level of the clavicle. The denervated pigeons were then left with other normal pigeons which were to be used as controls. They were fed on a normal diet of grains. At the end of the experimental period the bird was decapitated and pieces of the breast muscle were immediately excised and blotted to remove blood and used for the various studies.

GLYCOGEN: The excised pieces were quickly weighed on a Mettler balance and dropped into 2 ml. of hot 30% KOH. Glycogen in the digested material was estimated according to the method of Seifter et al. (1950) using anthrone reagent for the final colour development. The optical density of the solution was measured at 620mμ using a Bausch and Lomb "Spectronic 20" colorimeter. Glycogen content of the muscle is expressed as gm per 100 gm. wet muscle.

PHOSPHORYLASE: Phosphorylase activity was assayed by a modification of the method of Cori, Cori and Green (1943) as adapted by Cahill et al. (1957). Pieces of the breast muscle were homogenized in a chilled mortar to make a 2% homogenate in cold distilled water. Samples of 0.2 ml. of fresh homogenate were added to a chilled test tube containing 0.2 M. sodium citrate buffer, pH 5.9, 0.3 ml. of 0.154 M. potassium fluoride, and 0.05 ml. of 0.2 M. glucose-1-phosphate (L.Light and Co) as substrate. Controls were run with each experiment by adding 1 ml. of 10% trichloro-acetic acid (TCA) to the incubation mixture before the addition of the homogenate. The samples and the controls were incubated for 15 minutes at 30°C. and the enzyme activity in the samples was terminated by the addition of 1 ml. TCA. Blanks were prepared by adding 1 ml. of 10% TCA to the incubation medium. After incubation, the solutions were filtered into 10 ml. graduated test tubes with several washings. The inorganic phosphate liberated from glucose-1-phosphate through enzymic action was estimated in the filtrate by the method of Fiske and Subbarow (1925) and the optical density was read on a Klett-Summerson photo electric colorimeter using a 620 mμ filter. The readings for the controls were substracted from those of the samples to determine the amount of phosphate released by the enzymatic action. The enzyme activity is expressed as mg. phosphorous liberated per mg. protein of the muscle in 15 minutes at 30°C.

SUCCINIC DEHYDROGENASE: A piece of the breast muscle was homogenised in a chilled mortar with ice cold distilled water, and 5% homogenate was prepared after removing all the connective tissue. Succinic dehydrogenase activity was determined according to the method of Kun and Abood (1949) using triphenyltetrazolium chloride as electron acceptor. The incubation mixture of each experiment contained 0.5 ml. of 0.1 M. phosphate buffer, pH 7.4, 0.5 M sodium succinate, and 1 ml. of freshly prepared 0.1% triphenyl tetrazolium chloride in distilled water. To this, 1 ml. of muscle homogenate was added and incubated for 30 minutes at 37°C. Enzyme activity was terminated after 30 minutes by the addition of 7 ml. of acetone. Tubes were shaken well and centrifuged for 10 minutes at 3000 r.p.m. to obtain a clear supernatant containing extracted formazan. Absorption of the supernatant was read at 420 mμ on a Klett-Summerson photoelectric colorimeter. Readings were corrected for an incubated blank without substrate and the amount of formazan calculated from a standard graph. Enzyme activity is expressed as μg formazan formed per mg. protein of muscle in 30 minutes at 37°C. under aerobic conditions.

LIPASE: The method employed for the estimation of lipase activity was one adopted from Martin and Peers (1953) using a bicarbonate carbondioxide buffer system of pH 7.4 at 37°C. employing the Warburg apparatus as followed in this laboratory (George, Vallyathan and Scaria, 1958). The side arm contained 0.5 ml. of 4% (v/v) tributyrin in 0.0148 M. bicarbonate, emulsified by shaking with a drop of 'Tween 80'. Each reaction falsk contained 1.5 ml. of 0.025 M. bicarbonate buffer and 1 ml. of the enzyme solution being tested in the main chamber. The flask and the manometers were gassed for 3 minutes with a mixture of 95% nitrogen and 5% carbon dioxide. After an equilibration of 10 minutes in the Warburg constant temperature water bath, the substrate was tipped in and allowed to equilibrate for 3 minutes, to ensure complete mixing of the contents. The manometers were shaken at 120 oscillations / minute allowing an amplitude of 4 to 5 cm. per oscillation. The readings were taken at regular intervals for 1 hour. The enzyme activity in the muscle is expressed as micro liters of CO_2 / milligram protein / hour.

PROTEIN: Protein content of the homogenate was determined by the method of Gornall _et al_. (1949) using the biuret reagent .

Results

The results obtained for the normal as well as the denervated pigeon breast muscle are presented in Table I. It is evident from the results that the normal pigeon breast

TABLE 1

Changes in glycogen, phosphorylase, succinic dehydrogenase and lipase levels in pigeon breast muscle during denervation atrophy.

Days of atrophy	Glycogen mg./100 gm. wet muscle	Phosphorylase mg. phosphorus evolved/mg. protein /30 mits.	SDH. µg formazan evolved/mg. proten/30 mits.	Lipase µl CO_2 evolved /mg. protein/hr.	No. of experiments
7	0.620 ± 0.10	6.41 ± 0.43	2.562 ± 0.98	36.11 ±10.01	5
14	0.256 ± 0.16	0.35 ± 0.08	1.83 ± 0.43	39.59 ± 10.82	5
21	0.787 ± 0.44	0.30 ± 0.01	1.27 ± 0.31	28.25 ± 5.70	5
30	0.169 ± 0.03	0.298 ± 0.09	1.36 ± 0.42	33.38 ± 14.35	5
Normal	2.440 ± 0.56	1.067 ± 0.01	7.13 ± 0.68	39.02 ± 3.54	6

muscle possesses high succinic dehydrogenase and lipase activity. The glycogen content and phosphorylase activity are also moderately high. In the denervated muscle, the level of glycogen and all the other enzyme studied showed a decrease with the progress in atrophy in terms of the number of days after denervation.

A drastic fall in the glycogen level was observed on the 7th day with a very high phosphorylase activity. Thereafter the decrease in the glycogen and phosphorylase activity was gradual and proportional to each other. After 30 days of denervation, glycogen and phosphorylase levels were found to be the lowest. Succinic dehydrogenase and lipase showed a decrease with the increase in the number of days after denervation. Succinic dehydrogenase activity however, was very low even after 7 days of denervation. After 14 days the enzyme activity was nearly that of the normal muscle. Apart from the slight fluctuations, lipase activity did not show any sharp changes.

Discussion

It is well known that nerves exert a trophic influence on the muscles that they innervate and the severance of the nerve brings about atrophy of the muscle showing histological signs of degeneration, increase in the nuclear count , increase in connective tissue and fat and many biochemical changes. It is also well known that the disuse of the muscle brings about changes which are by and large similar to those after denervation. In the present study normal pigeons were used as controls as against the common practice of using the contralateral muscle as control to avoid any changes which may be caused as a result of inactivity. In the previous chapters it has been shown that each type of muscle fibre showed a rapid decrease in the activities of the enzymes which were normally higher in that particular fibre type. Gradually not only the enzymatic differences between the various fibre types decreased, but the glycolytic dependent ones geared to an oxidative metabolism and the aerobic dependent ones to an anaerobic metabolism for their energy transformation. The observation, that the red fibres are loaded with glycogen and phosphorylase and the white fibres with increased number of mitochondria and oxidative enzymes, forms the basis of the above assumption. Thus there is no doubt that in denervation atrophy occurs a metabolic disturbance . However, it is not clear whether the morphological changes result from this or vice versa. The possibility that the metabolic disturbance is due to a lack of the metabolites, can be over ruled since denervation in no way hampers blood supply (Bass and Hudlicka, 1961).

The low level of glycogen observed in the present study though tallies with the observations of Hines and Knowlton, (1935) and Lazere <u>ét al</u>. (1943), is not in agreement with the histochemical data (Chapter 6). This apparent inconsistency with regard to the histochemical data is not to be regarded as controversial since the red fibres are richer in glycogen synthetase for the conversion of glucose to glycogen than the white fibres during the inactive state (Bocek and Beatty, 1966). Glycogen thus formed in the red fibres is not stored, instead may be undergoing faster breaking down so as to yield energy. This is further evident from the increase in phosphorylase seen in the red fibres (Chapter 6).

It has been reported by Hudlicka and Bass (1961) that the denervated muscle showed a high glucose and oxygen consumption. The immobilized muscle was also shown to have a higher R.Q. value (Chapter 5). During functional activity lipids are utilized by normal pigeon breast muscle and other mammalian skeletal muscles (George and Jyoti, 1955, 57,; George and Vallyathan, 1964; Fritz, 1958; Neptune et al, 1963). In the case of atrophy, lipid utilization is seen to be defective (Chapter 5) and the energy source viz. carbohydrates which are generally utilized mainly via anaerobic glycolysis in the normal muscles, is utilized through the oxidative pathway. Evidence for the increased activity of the oxidative, pathway is seen from the increase in O_2 consumption (Hudlicka and Bass, 1961) and increase in the shunt enzymes (Chapter 6).

From the histochemical data on the denervated muscle (Chapter 6) and the quantitative assay of the muscle under disuse (Chapter 5) the probability of increased fat synthesis during this period cannot be over ruled. The increased activity of the pentose cycle enzymes observed in the denervated muscle (Chapter 6), the inability of the disused muscle to oxidise fatty acids, higher R.Q.values (Chapter 5) and the high activity of the enzyme dihydrolipoic dehydrogenase noted in the dystrophic human muscle (Chapter 8) strengthen such a possibility.

When the metabolic disturbance of the muscle is viewed from this angle, the increased level of phosphorylase observed immediately after denervation in the present study is not surprising . The increased phosphorylase level only denotes faster utilization of the stored glycogen during this period and the depletion thereafter, corresponds to the decreased glycogen content. The present investigation closely agrees with that of Matsui and Kuriaki (1959), Humoller _et al._ (1951), and Huls and Leonard, (1961); but disagrees with that of Arson and Volk (1957), who reported a decrease in phosphorylase activity immediately after denervation.

Since the low level of succinic dehydrogenase of the denervated muscle denotes its reduced oxidative metabolism, the dependence of the latter on the innervation can be doubted. However, such an assumption has no validity as the immobilized muscle also showed a low level of that enzyme (Vallyathan, 1963). The conclusion to be drawn from these findings is that the metabolism of the muscle is geared by its functional state.

The consistent low lipase level observed in the present study appears to be rather intriguing as it was contrary to expectation. A high fat content together with increased lipase activity have been observed during the first week of immobilization atrophy (George and Vallyathan, 1964). In the present study too, such a high level must have occurred during the first week following denervation. It is quite unlikely that the occurrence of high enzyme level does not exist in the muscle during this period in view of the present evidences. But the failure to obtain such an observation is due to the fact that no investigation could be made prior to the 7th day following denervation in the present study.

It may be concluded that both in the immobilized and the denervated muscles the rate of atrophy was of the same speed and magnitude during the initial stages. But thereafter, the denervated muscle continued to atrophy, whereas the muscle inactivated by plaster cast began to regain its normal metabolism.