Transmural Gradient of Glycogen Metabolism in the Normal Rat Left Ventricle

V. De Tata, C. Bergamini*, Z. Gori, T. Locci-Cubeddu, and E. Bergamini

Istituto di Patologia Generale, II Cattedra, Università di Pisa, Via Roma 55, Scuola Medica, I-56100 Pisa, Italy

Abstract. The changes of glycogen metabolism with the location of tissue within the ventricle wall have been explored in the rat myocardium. The hearts were cut in 100 μm thick serial sections and all sections were analyzed for their content in glycogen, glucose-6-phosphate, UDPG and glycogen enzymes and for glucose incorporation into glycogen and for the 2-deoxyglucose uptake after the intravenous injection of the ^{14}C-labelled sugars.

The rate of glycogen turnover was significantly higher in the subendocardial myocardium ($P < 0.01$) and the levels of glucose-6-phosphate and the total (i.e. a+b) activity of glycogen phosphorylase were significantly higher in the subepicardial tissue ($P < 0.01$ in both instances). No significant transmural gradient of UDPG was found and transmural changes of total (i.e. I+D) synthase activity were barely significant.

These changes in glycogen metabolism may be related to regional differences in the cardiac work load and to a differentiation of the subendocardial and subepicardial heart fibers.

Key words: Subendocardial − Subepicardial − Glycogen − Glycogen metabolism − Myocardial metabolism

Introduction

Evidence supporting the hypothesis of different nutrition and metabolism of the subendocardial region of the heart was provided in 1964 by two independent studies. Kirk and Honig (1964b) demonstrated nonuniform gradients in blood flow and lower tissue oxygen tension in the subendocardium. In the same year, Jedeikin showed that there were higher levels of glycogen and increased phosphorylase activity in the subendocardium compared to the outer layers of the heart.

Later studies by other investigators showed increased glycolytic enzyme activity (Lundsgaard-Hansen et al. 1967) and increased production of lactate during ischemic work (Dunn and Griggs 1975) in the inner layers. With regard to glycogen, the existance of a diminishing gradient of concentration was confirmed in the dog, proceeding from the endocardium to epicardium by Allison and Holsinger (1977) but not by Todd et al. (1979).

These controversial reports do not answer the question as to whether during normal contraction the glycogen of a particular region is utilized preferentially over any other. This is of great interest in view of the essential role of glycogen in muscle contraction (see Bergamini et al. 1980; Villa-Moruzzi et al. 1981).

Materials and Methods

Animals

Young adult (170−180 g b.w.) female albino rats of Sprague-Dawley strain were used. Different groups of animals were used for each parameter measured. In all cases a 12 h fasting preceded experimentation. Anesthesia was performed by injecting Nembutal (5 mg/100 g b.w., intraperitoneally). [14](C)-uniformly-labelled glucose (S.A. 3 mCi/mmole) was dissolved in saline and injected intravenously in the tail (10 μCi/100 g b.w.) 10 min before sacrifice. [14](C)-uniformly-labelled 2-deoxyglucose was diluted in saline and injected at the same dosage and time by the same route.

Animals were killed by causing a cardiac arrest with 1.0 ml cold 150 mM KCl injected intravenously. The arrested heart was rapidly removed and dropped in ice (in less than 10 s) and a piece of the left ventricle was excised and frozen between two aluminium blocks at −30°C, without any compression. The frozen tissue was mounted (the epicardium upside) and cut in 100 μm thick sections in a cryostat. To get the needed amount of tissue, two adjoining sections were combined. During these procedures temperature was −25°C.

Determination of Glycogen

Sections were dropped in centrifuge tubes containing 0.5 ml 4 N NaOH and the tubes were kept in boiling water for 30 min. Aliquots were used to assay the protein content by the Lowry procedure (Lowry et al. 1951). Then, glycogen was purified according to Hassid and Abraham (1957) and assayed spectrophotometrically by the anthrone procedure. Results are given as mg glycogen/g wet weight. Wet weight always was calculated from protein content as follows: g wet weight $= K \times$ g protein (the factor $K = 6.25$ was found empirically).

Incorporation of [14](C)₆-Glucose Into Glycogen

In order to test at least two different experimental conditions, experiments with labelled glucose and 2-deoxyglucose were performed by using both anesthetized and "unanesthetized" rats (i.e. rats not treated with pentobarbital and free in their

cages during the whole experiment until they were killed by the KCl infusion). The heart sections were processed as described (see: determination of glycogen) but carrier glycogen was added to the hydrolyzate and glycogen was then purified as described (Bergamini et al. 1969). The final sediment was dissolved in 0.4 ml distilled water and an aliquot (0.3 ml) was applied onto 3MM Whatman filter paper disks and dried and the radioactivity was counted in a liquid scintillation counter with a standard liquid scintillation fluid (Liquifluor) to an error less than 3%.

2-Deoxyglucose (2-dGlc) Uptake and Phosphorylation

Sections were dropped in centrifuge tubes containing 0.5 ml distilled water. Sugar was then extracted at 100° C for 10 min. Total 2-dGlc and non phosphorylated 2-dGlc were determined by counting the radioactivity of aliquots of the extract respectively before and after treating it according to the Somogyi procedure — as in Tietz (1976) — to an error less than 3%. The precipitated proteins were digested in NaOH 4 N and were assayed as described. Results are given as counts/mg wet weight.

Assay of the Activities of Glycogen Metabolizing Enzymes

Sections were dropped in centrifuge tubes containing 0.5 ml distilled water and enzymes were extracted at 0° C for 10 min with occasional shaking and stirring with a glass rod. Assays were performed on aliquots of the extract without any need of centrifugation.

The total (i.e. I+D) glycogen synthase (GS) activity was measured as the amount of labelled glucose incorporated into glycogen from UDP-$^{14}C_6$-glucose during a 15 min incubation at 30° C by the filter paper method of Thomas et al. (1968). The total glycogen phosphorylase activity (GPho) was assayed from the amount of inorganic phosphate released from glucose-1-phosphate in the presence of AMP during a 10 min incubation at 37° C according to Turner and Leonard (1969).

Other Assays

Glucose-6-phosphate, UDPG and lactate were assayed on perchloric acid extracts of 2 heart sections after neutralization with solid $KHCO_3$. The assays were performed by enzymatic techniques (see Bergmeyer 1974) by using a Perkin Elmer Spectrofluorometer.

Chemicals

Oyster glycogen, glucose-1-phosphate, glucose-6-phosphate, UDPG and NADP were products of Sigma Chem. Co. (St. Louis, MO, USA). Crystalline glucose-6-phosphate dehydrogenase and UDP-glucose dehydrogenase were purchased from Boehringer (Mannheim, FRG). $^{14}(C)_6$-labelled glucose was obtained from the Radiochemical Centre (Amersham, GB) and 2-dGlc from New England Nuclear, Dreieich, FRG. UDP-$^{14}C_6$-glucose was prepared according to Thomas et al. (1968).

All products used were of analytical grade.

Statistical Analysis

Statistical significance was tested by using the Student t-test and one-way analysis of variance.

Results

1. Glycogen Distribution in the Layers of the Myocardium

In all the figures presented, the horizontal axis represents a progression of values for successive serial sections. The first and last values are always those of the subepicardial and subendocardial layers respectively. It should be mentioned here that these two layers contain larger amounts of connective tissue.

Figure 1 summarizes the results of determination of glycogen distribution throught the walls of the rat left ventricle. The levels of glycogen are much higher than that previously reported (Jedeikin 1964) and are very similar in all myocardial layers (the slight increase in the subendocardium is not significant). This is unlike the data of Jedeikin (1964). To comment this discrepancy, we may mention that the data published by Jedeikin seem to be greatly affected by the experimental procedure at sacrifice (ether or pentobarbital anesthesia or decapitation) and that Gaesser and Brooks (1980) have concluded that the glycogen concentrations in the cardiac muscle may be more attributable to the method of killing of the rats than to the effects of the experimental protocols. In our experiments, inconvenience has been circumvented in part by injecting cardioplegic solution intravenously.

2. Distribution of $^{14}C_6$-Labelled Glycogen in the Layers of the Myocardium after an Intravenous Injection of Labelled Glucose

In the skeletal muscle, an apparent steady state of glycogen levels may result from very different dynamics of glycogen metabolism. In fact, glycogen levels are quite stable, whereas glycogen turnover can change dramatically even at very light work loads (Villa-Moruzzi et al. 1981). Therefore, we decided to explore the incorporation of glucose into heart glycogen during a 10 min exposure to intravenously injected $^{14}C_6$-labelled glucose (Fig. 2).

A highly significant ($P<0.01$) gradient of glucose incorporation was found in the walls of the left ventricle (higher values were found in the subendocardial layers). It should be emphasized that similar patterns of glucose distribution were observed in the anesthetized and in the unanesthetized rats
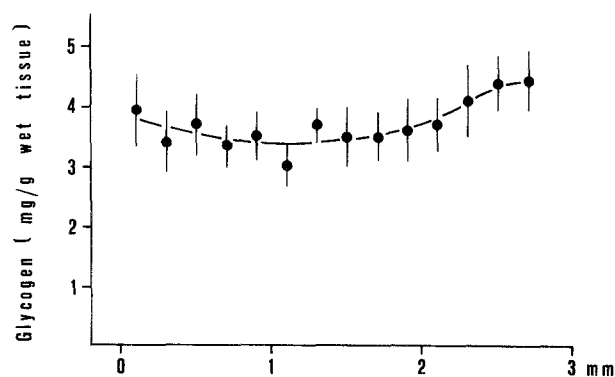
Fig. 1. Distribution of glycogen across wall of the left ventricle of rat heart. On the abscissa: mm of myocardial tissue, from epicardium (left) to endocardium. Means of 6 cases are given. Vertical segments represent $2 \times$ SEM. No significant difference in glycogen content can be observed among the different myocardial layers

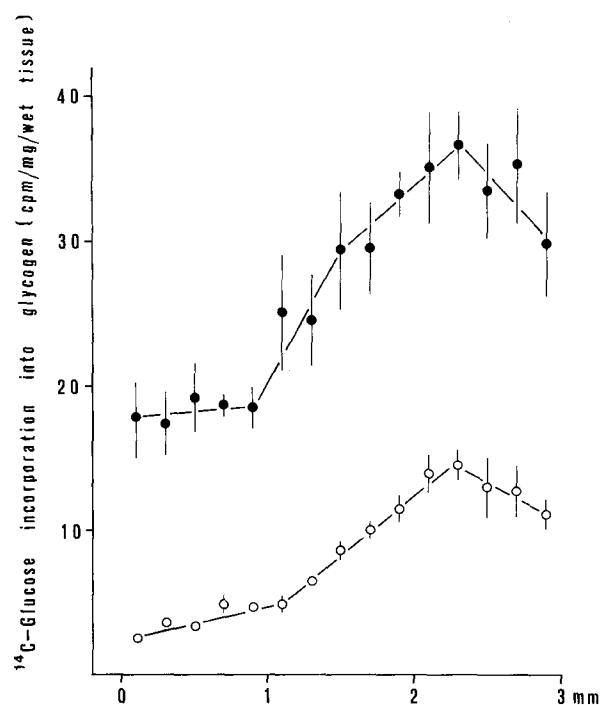Fig. 2. Glucose incorporation into glycogen in vivo in different myocardial layers of the rat left ventricle. 10 µCi $^{14}C_6$-labelled glucose/100 g body weight were injected in the tail vein (in 0.2 ml saline) of anesthetized (●) or of unanesthetized (○) rats 10 min before sacrifice. Glycogen was purified and the radioactivity was counted as described in the text. Results are given as counts/mg wet cardiac tissue. Means of 4 cases are given. Vertical segments represent 2 × SEM. On the abscissa: mm from epicardium. Statistical analysis (F-test) has shown that the effect of anesthesia and differences among the myocardial layers are highly significant ($P < 0.01$). Interaction is not significant
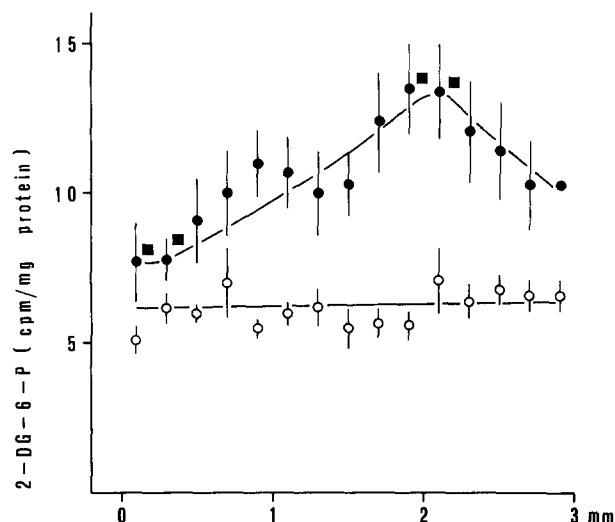

Fig. 3. Accumulation of 2-deoxyglucose-6-phosphate in different myocardial layers of the left ventricular wall of the rat heart. Experiment was performed as described in Fig. 2 with the same amount of radioactivity for the same time. Anesthetized (●) and unanesthetized (○) rats were used. The sugar phosphate was extracted and purified from the free sugar as described in the text. Results are given as counts/mg wet cardiac tissue. Means of 4 cases are given. Vertical segments represent 2 × SEM. On the abscissa: mm from epicardium. The effect of anesthesia is highly significant ($P < 0.01$). Differences among myocardial layers are significant ($P < 0.01$) in the anesthetized rats only. The symbol (■) denotes a statistically significant difference ($P < 0.05$; Bonferroni's t-multiple test)

(interaction is not significant). We should comment also that the incorporation was significantly different ($P < 0.01$) in the two groups of rats (i.e. a higher per cent value of the injected glucose was incorporated into the heart glycogen in the former case). This difference may be due to additional factors besides the different work load. For example, stress due to handling and to injections is different in anesthetized and in the unanesthetized rats. As an additional factor, the per cent uptake of the labelled glucose by different tissues (e.g. by the skeletal muscle, see Villa-Moruzzi et al. 1981) is lower in the anesthetized animals.

3. 2-dGlc Uptake and Phosphorylation

In the rat heart, the rate of the glucose uptake may be the factor limiting the rate of sugar metabolism (Morgan et al. 1961). The phosphorylation (by hexokinase) and the transfer into glycogen of the glucose units (by glycogen synthase) are the other limiting steps along the metabolic path leading to glycogen synthesis (see e.g. Bergamini et al. 1969). To monitor the accumulation of 2-dGlc-6-phosphate after the injection of trace amounts of the labelled sugar (this sugar is transported and phosphorylated like glucose but it is not metabolized further, Kipnis and Cori 1959) may provide a good index of the hexokinase activity in the intact tissue (Karpatkin et al. 1966) and help to evaluate the contribution of the first two metabolic steps to the increased glucose incorporation.

In Fig. 3 we have depicted the distribution of 2-dGlc-6-phosphate (as counts/mg wet tissue) in the different layers of the myocardium after the exposure to a dose of radioactivity and time like those used in the glucose incorporation studies. The amount of the phosphorylated radioactivity accumulated in the myocardium is significantly larger in the anesthetized rats (but difference is smaller than that observed for glucose incorporation). A transmural difference in 2-dGlc phosphorylation is observed in the anesthetized rats because of the lower subepicardial values. Similar findings were reported in the dog (L'Abbate et al. 1979, 1981). At present, it is not possible to state whether this effect should be attributed to a lower dependence on glucose of the subepicardial layers or to some action(s) of pentobarbital per se. In any case, we can conclude that a higher sugar uptake and phosphorylation does not contribute to the higher subendocardial incorporation of glucose into glycogen at least in the unanesthetized rats.

4. Levels of Glucose-6-Phosphate and of UDPG in the Rat Myocardium

The levels of glucose-6-phosphate and of UDPG within the muscle cells can greatly affect the activity of the glycogen synthetizing enzyme (Piras et al. 1969; Roach et al. 1976). We found significant differences in glucose-6-phosphate content in the different myocardial layers ($P < 0.01$) (Fig. 4). However, in this instance the transmural gradient is reversed, higher levels having been observed in the subepicardial tissue. With regard to UDPG, no definite change was observed in its content. We may conclude that allosteric regulation of the glycogen synthase activity by sugar phosphate cannot explain the larger incorporation of glucose in the subendocardial tissue.
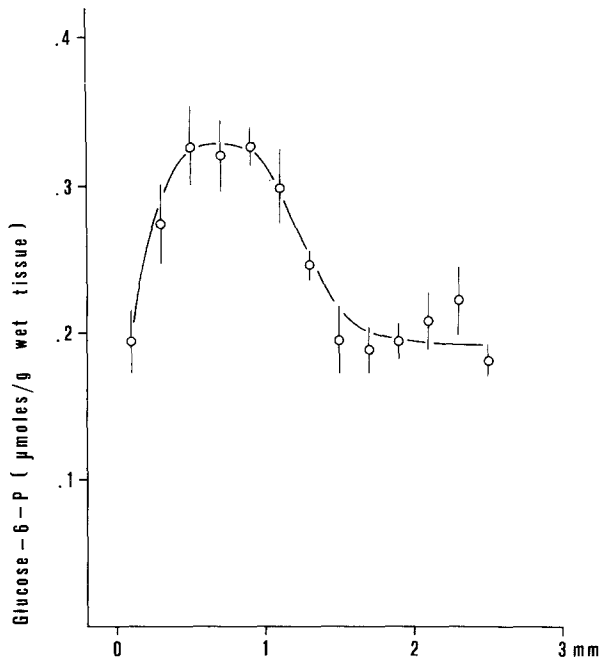
Fig. 4. Distribution of glucose-6-phosphate in the different myocardial layers of the left ventricle of the rat heart. The sugar phosphate was extracted and assayed as described under Materials and Methods. Results are given as µmoles of sugar phosphate/g wet cardiac tissue. Means of 6 cases are given. Vertical segments represent 2 × SEM. On the abscissa: mm from epicardium. The differences among the myocardial layers are highly significant ($P<0.01$). On the same extracts we have assayed also UDPG content. The distribution of the sugar nucleotide was quite uniform in the different myocardial layers
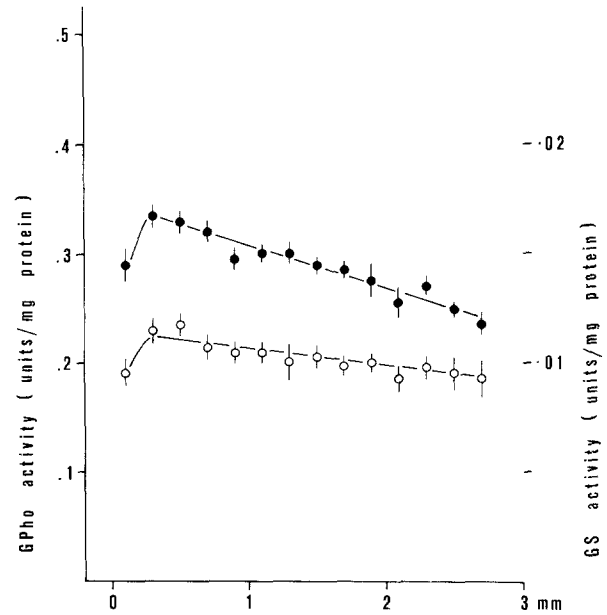
Fig. 5. Glycogen phosphorylase (●) and glycogen synthase (○) distribution across wall of the left ventricle of the rat heart. The enzyme activities were assayed as described under Materials and Methods. Results are given as units (µmoles/min) per mg of soluble protein in the extract (in the case of glycogen synthase, scale has been expanded 20 times; look at wright). On the abscissa: mm from epicardium. The transmural gradient of the enzyme activity is highly significant in the case of glycogen phosphorylase ($P<0.01$) and barely significant ($P = 0.05$) in that of glycogen synthase

5. Distribution of Glycogen Enzymes in the Rat Myocardium

Figure 5 presents data on the total glycogen synthase and glycogen phosphorylase activities across the wall of the rat left ventricle. A highly significant ($P<0.01$) linear decrease in the tissue phosphorylase activity was found in layers proceeding from epicardium to endocardium. This is unlike the single representative experiment on the total phosphorylase activity in the rabbit heart shown in Jedeikin's paper (Fig. 5) (1964). We have no explanation for this discrepancy. On the other hand, total glycogen synthase activity exhibits much smaller (and barely significant, $P = 0.05$) changes. In these fully relaxed hearts, the per cent active forms of the two enzymes [i.e. the phosphorylase $a/(a + b)$ and the glycogen synthase $I/(I + D)$ ratios] were constant across the cardiac wall.

Discussion

In these experiments, the regional distributions of substrates and enzymes across the cardiac wall were assessed by cutting the tissue in serial sections of a constant thickness (100 µm). Thus, at variance from all the previous reports, here the metabolic and enzyme changes can be correlated with the location of the tissue within the cardiac wall precisely. On the other hand, the use of this technique is not compatible with that of quick-freezing procedures (see e.g. in Griggs 1979), since samples have to be excised and then to be frozen without compression (to preserve the thickness of the wall) at a temperature optimal for the subsequent cutting (i.e. $-30°$ C). We may mention that the rat myocardial wall is relatively thin

(2.5–3.0 mm) and by using large precooled aluminium blocks to freeze tissue we could go through the whole procedure in about 30 s. This may appear to be a quite long time. However, it should be remembered that the hearts were fully relaxed and cold due to KCl cardioplegia at $0°$ C and that relaxation can be expected to lower the consumption of energy hundreds of times with respect to contraction (see e.g., Wilson et al. 1981). In effect, we found that lactate concentration in the whole left ventricle wall ranges between 0.7 and 2.0 µmoles/g w.w. in the anesthetized rats and these values are not much higher than those reported in literature (see Griggs 1979). No significant transmural gradient of lactate concentration was observed. The phosphorylase ($a/a + b$) ratio averaged $29 + 3.7\%$ ($n = 5$). We may conclude that during the removal and freezing of the heart, the breakdown of glycogen was very low (less than 1 µmole glucose/g wet tissue, i.e. less than 4 % of the total glycogen content) and that no gradient of breakdown can account for the observed transmural gradient of glucose incorporation.

Our results show that in the different myocardial layers the apparent steady state of glycogen levels results from very different dynamics of glycogen metabolism. On the other hand, differences in the rate of glucose incorporation and similarities in the concentrations of glycogen necessarily imply that compensations occur in the different layers due to differences in glycogen breakdown.

These same conclusions have been reached with regard to glycogen metabolism in fast and slow muscles at different work loads (Villa-Moruzzi et al. 1981). In this latter case the changes of glycogen turnover reflected changes in the work

load. It was concluded that in the contracting tissues glycogen should not be considered merely as a store of energy to be used or not according to occasional needs (as in Lehninger 1975). In line with the most modern understanding of the role of Ca^{2+} in the control of glycogen breakdown, it was suggested that glycogen is always used during contraction (Bergamini et al. 1980; Villa-Moruzzi et al. 1981) to help to "buffer" the changes of ATP and ADP levels, together with creatine phosphate (see Wilson et al. 1981).

In this perspective, the transmural gradient of glycogen turnover should be considered to be the consequence of a transmural gradient of the load of work. Unlike the case of the skeletal muscle, in all myocardial layers all muscle fibers are bound to contract with the same frequency and differences in the work load may be related only to differences in the generated intramyocardial pressure. In this respect, no data are available on the rat heart at present. However, it might be worth mentioning that biophysical evidence of a non uniform transmural force in the rhytmically contracting left ventricle have been presented by Armour and Randall (1971) and by Kirk and Honig (1964a). Furthermore, the availability of micromanometers has made possible the measurements of intramyocardial pressure at different depths within the ventricular wall in the dog and it has been shown that pressure is remarkably higher in the subendocardial layers than in the subepicardial ones during systole (Stein et al. 1980a), that the transmural gradient has an opposite direction during diastole (Stein et al. 1980b) and that this latter difference disappeared following cardiac arrest (Stein et al. 1980b). Our data indicate that the subendocardial layers may be subjected to a larger work load during the cardiac revolution. Incidentally, in hearts beating in frankly ischemic conditions a greater elevation of lactate was observed in the inner layers and it was concluded that the inner ventricular layer is more susceptible than the outer layer to an imbalance between oxygen supply and demand when the autoregulatory capacity of the coronary circulation is exceeded (Griggs 1979).

Our results show that lower glycogen phosphorylase levels are found in the rat subendocardial muscle tissue. In the skeletal muscle, lower glycogenolytic activities (see e.g. Villa-Moruzzi et al. 1979) and higher glucose incorporation into glycogen were found in the slow muscle as compared with the fast muscle (see Villa-Moruzzi et al. 1981). Recent studies on myosin polymorphism in cardiac muscle have shown that the number of ventricular fibers stained selectively by antiguinea pig soleus myosin immunofluorescent antiserum in the rat heart increased with age (Gorza et al. 1981). Regional variations in ventricular isomyosin distribution have been reported in the left ventricle of the rabbit heart (Sartore et al. 1981). The problem still is unanswered, whether a similar distribution of phosphorylase-poor fibers may lead to the metabolic differences observed here. Also, it should be mentioned that quantitative ultrastructural differences have been found in hearts from various species and have been shown to be the structural correlates for different physiological properties (Schaper et al. 1981). As a final comment, biochemical evidence that myocardial oxygen consumption may be larger in the inner than in the outer layers has been produced (Holtz et al. 1977; Weiss et al. 1978) and triglycerides and myoglobin levels may be higher and the mitochondrial populations may be different in the subendocardial layers of the dog heart (Whitty et al. 1968).

On the whole, all these data indicate that larger amounts of glycogen are used by the subendocardial myocardium (where energy consumption may be greater) and that muscle differentiation probably occurred to cope with these demands. Perhaps, this may provide an additional insight into the mechanisms for the greater susceptibility of the subendocardium to ischemic injury.

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