GLYCOGEN METABOLISM IN RED AND WHITE MUSCLE

by

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INTRODUCTION

I. Observations on Differences in Structure and Function of Muscle

Movement in living organisms is associated with life. Each motile species has developed organs of movement which are adapted to the specialized needs of the organism for survival and propagation of its kind. Single-celled animals are propelled by means of flagella and cilia. Higher aquatic animals move by contracting certain structures thereby pushing themselves about by expelling water in a primitive jet propulsion mechanism. Certain mollusks utilize this principle and achieve movement by rapidly snapping shut their shells by contraction of the catch muscles. In higher vertebrates movement is associated with the development of more specialized organs enabling the animal to travel great distances in search of food and survival.

It is evident in higher animals that the increase in complexity of movement is associated with a concomitant increase in the numbers and specialized functions of muscles. The classic division into cardiac, smooth and skeletal muscle is an expression of the ultimate diversification of structure and function achieved in the evolution of mammalian muscle.

Striated skeletal muscle has long been known to exist in at least two forms, the red or dark and the white or light muscle. Certain muscles are of uniform color macroscopically, but others such as the semimembranosus, the semitendiosus, or the gastrocnemius of the rat

consist of darker and lighter areas. It is a fundamental observation that white muscle is associated with quick, powerful motion of short duration and dark muscle with slower, prolonged function. It has been assumed that differences in function are reflected in differences in mechanism of energy production for muscle contraction. White muscle is considered to have a well-developed glycolytic system and poor oxidative mechanism. Red muscle, capable of prolonged operation, has high oxidative activity. Because of historical beginnings in investigation of the process of fermentation, glycolysis has been associated with anaerobic metabolism. It is not surprising then to find that muscle contraction was associated with glycolytic processes in early investigations. With the discovery of the citric acid cycle as the focal mechanism of oxidative degradation of carbohydrate for the production of energy for muscle work, a natural duality arose in explaining the mechanisms of energy production--anaerobic glycolysis versus oxidation. While it is true that anaerobic glycolysis can supply a limited amount of energy for brief periods of activity, the metabolism of glucose usually proceeds through the glycolytic pathway and then enters the oxidative steps of the citric acid cycle.

It seemed of interest, therefore, to attempt to determine the relative importance of the glycolytic pathway in red and white muscle, both _in vivo_ and _in vitro_. This paper reports investigations into the metabolism of glycogen in these two types of muscle fibers. It is proposed in the introduction to outline briefly some of the historical background on red and white muscle differentiation and metabolism, to indicate the chemical and histochemical differences which have been

reported, and to discuss the background of the enzymatic mechanisms of glycogen synthesis and degradation.

II. Early Studies of Red and White Muscle

The work of numerous investigators of the 18th and 19th centuries established the fundamental differences in histology, physiology and chemistry between red and white muscle. Although access to individual papers is limited because of the age and variety of journals, it seems important to review some of the most important work of this period as cited by Needham[83], Bullard[16], and Björk[9].

Among the early speculations about the nature of the color of red muscle was the work of Boerhave[15] in 1739 who thought that the red color, due to the presence of blood, could be removed by washing the blood away. Hildebrandt[43] in 1789 maintained that the coloration is due to an increase in the amount of blood vessels (the red being the more vascular). In 1850 Kölliker[54] denied that the coloration is due to numbers of blood vessels but rather to a pigment associated with the muscle fibers themselves. Kühne[59] in 1865 carried out experiments with perfused muscle and showed that the coloration is due to a pigment which he called hemoglobin. Kühne found that the oxy, reduced, and hematin compounds of muscle and blood have the same spectroscopic characteristics. Although he noticed a spectroscopic difference in the carbon monoxide derivatives of muscle and blood pigments, he attached no significance to this fact and missed differentiating between myoglobin and hemoglobin.

Lankester[60] in 1871 recognized that pigmented fibers are found in muscles which are active for prolonged periods. He observed that

in lower animals nearly all muscles except heart muscle, which is constantly active, are white, whereas in higher mammals nearly all muscles are pigmented. Exceptions to these observations were noted, however.

Ranvier[101] was the first to obtain experimental evidence that the behavior of red and white muscle is different. In support of Lankester, Ranvier found that the contractile response to stimulation is of shorter duration in white muscle than in red. More stimulations per second are required to produce smooth continuous contraction of white muscle than of red. These observations were made with rabbit and fish muscle and indicated a functional difference between the two types of muscle.

Grützner[39] observed that the muscles were made up of two types of fibers, one small and dark and the other large and pale or white. All human muscles have both types of fibers. Grützner believed that the dark fibers, which give coloration to red muscle, contain a greater number of 'granules' than do the white fibers. These 'interstitial granules' of Kölliker were studied by Retzius[102] who determined that they differ from fat droplets. He called these structures sarcosomes, but they are now more commonly called mitochondria. Grützner[39] believed that all fine, granular (dark) fibers, whether pigmented or not, correspond to red slowly contracting fibers of the rabbit and that the larger clear fibers correspond to fibers of the more rapidly moving white muscle. Knoll[52] also believed that dark fibers contain more interstitial granules. In addition, Knoll observed that red fibers contain more sarcoplasm and active muscles contain more dark

fibers[53].

Rollett[107] in 1890 published several studies on the histologic appearance of muscle fibers in a wide variety of species of insects, fish and mammals, and concluded that all muscles cannot be accurately catagorized as red-slow and white-fast acting. In particular, he noted that insect striated muscles are generally not pigmented and vary in slow-fast contractile functions.

Bullard[16], using cross-sections of frozen, unfixed muscle, with dark field illumination, observed that dark small fibers contain more 'interstitial granules' and more fat which stained with Sudan IV than do the light fibers. He also found that at the age of 7-8 months the human fetus contains clearly marked dark and light fibers. Bullard cautioned against the synonymous use of the terms red and dark and light and white. He states that in winter, the white muscles of the frog show a large percentage of very dark fibers (dark defined as having a high content of granules) and that dark fibers do not necessarily give a red color to muscle.

These descriptive studies on red and white muscle were followed by numerous investigations which attempted to show chemical differences between the two types of muscle. In 1926, Dorothy Moyle Needham[83] published a review on red and white muscle which summarized much of these early metabolic studies in addition to reviewing histological and physiological differences. Significant among the chemical differences found by early experimenters were that red muscle produces less lactate, contains less creatine and inorganic phosphate, has a higher cholesterol and phosphatidyl phosphorous content,

oxidizes succinic acid at a higher rate, and produces more carbon dioxide and consumes more oxygen than does white muscle.

Parnas and Wagner[92] in 1914 showed that lactic acid is derived from muscle glycogen, and Meier and Meyerhof (1924)[70] showed that the free energy derived from the formation of lactate could provide the energy necessary for muscle work. Although the breakdown of glycogen to lactic acid had been investigated in white muscle, Needham remarks "No correlation of glycogen loss and lactic acid formation has ever been worked out for red muscle...."[83].

III. Historical Development of Energy Producing Systems

A. Glycolytic Pathway

The period 1926-1931 saw rapid development in understanding the chemistry of metabolism. Parnas[91], in 1932, reported that among important advances of this period was recognition of the metabolic roles for creatine phosphate, adenyl pyrophosphates, and phosphorylated hexoses, the latter being found as intermediates in the production of lactic acid from glycogen. One of the most revolutionary discoveries was that of Lundsgaard[71] who demonstrated that muscle is capable of contraction without lactate formation so long as creatine phosphate stores are available. Previously, all muscle metabolism studies had been strongly influenced by the accepted theory that muscle contraction is directly linked to lactic acid production.

The principal features of the metabolic breakdown of glycogen in glycolysis (the Embden Meyerhof pathway) were elucidated in the period 1933-1939[78], although phosphorylated esters had been recognized for some years as being produced and utilized in the glycogen-

to-lactic acid process of both yeast extracts and muscle minces. With discovery that creatine phosphate reserves are utilized to maintain the ATP content of muscle (Lohmann)[70], the central position of ATP in relation to muscle contraction was indicated.

B. Oxidative Pathway

Although muscle contraction was studied relative to anaerobic glycolytic mechanisms for many years, many observations on respiration and on oxidative metabolism of carbohydrates by muscle had also been made. Among pioneering investigators in this area were men such as Thunberg, Warburg, Wieland, A. Szent Györgyi, Batelli, Stern, and Knoop[57]. Synthesis of the results of these and other investigators was achieved by Krebs and Johnson in 1937[56] with demonstration of the constituents and sequence of reactions in the citric acid cycle. When it was recognized that oxidative mechanisms are the principal source of ATP in muscle, the major section of the energy producing mechanism in muscle fell into place. Glycolysis was linked to the citric acid cycle through pyruvate, which, when converted to acetyl coenzyme A, condenses with oxaloacetate to form citrate. Oxidation of the reduced pyridine nucleotide in the electron transport system is the immediate source of ATP.

The relative importance of anaerobic glycolysis and the oxidative pathway may be indicated as follows. Muscles incubated in nitrogen contract with the formation of lactic acid at the expense of glycogen breakdown. This process yields a net of 2 moles of ATP per glucose converted to lactate. See Figure I. Creatine phosphate serves as an immediately available energy store to prolong muscle activity. On

aerobic recovery, about 20% of the lactate formed, or its carbohydrate equivalent, is oxidized via the TCA cycle to furnish energy for resynthesis of CrP and glycogen stores[77].

However, aerobically, glucose or glycogen is metabolized via the glycolytic system without net lactate production and the net yield of ATP/mole of glucose converted to pyruvate is 8 moles (3 moles produced per triose by oxidation of DPNH via the electron transport system). From Figure II it is evident that per mole of pyruvate oxidized in the citric acid cycle, 15 moles of ATP are formed. The over-all yield of ATP from the oxidation of 1 mole of glucose is therefore 38 moles of ATP, of which 8/38, or about 21%, is formed by glycolytic mechanisms.

IV. Metabolic Differences in Red and White Muscle

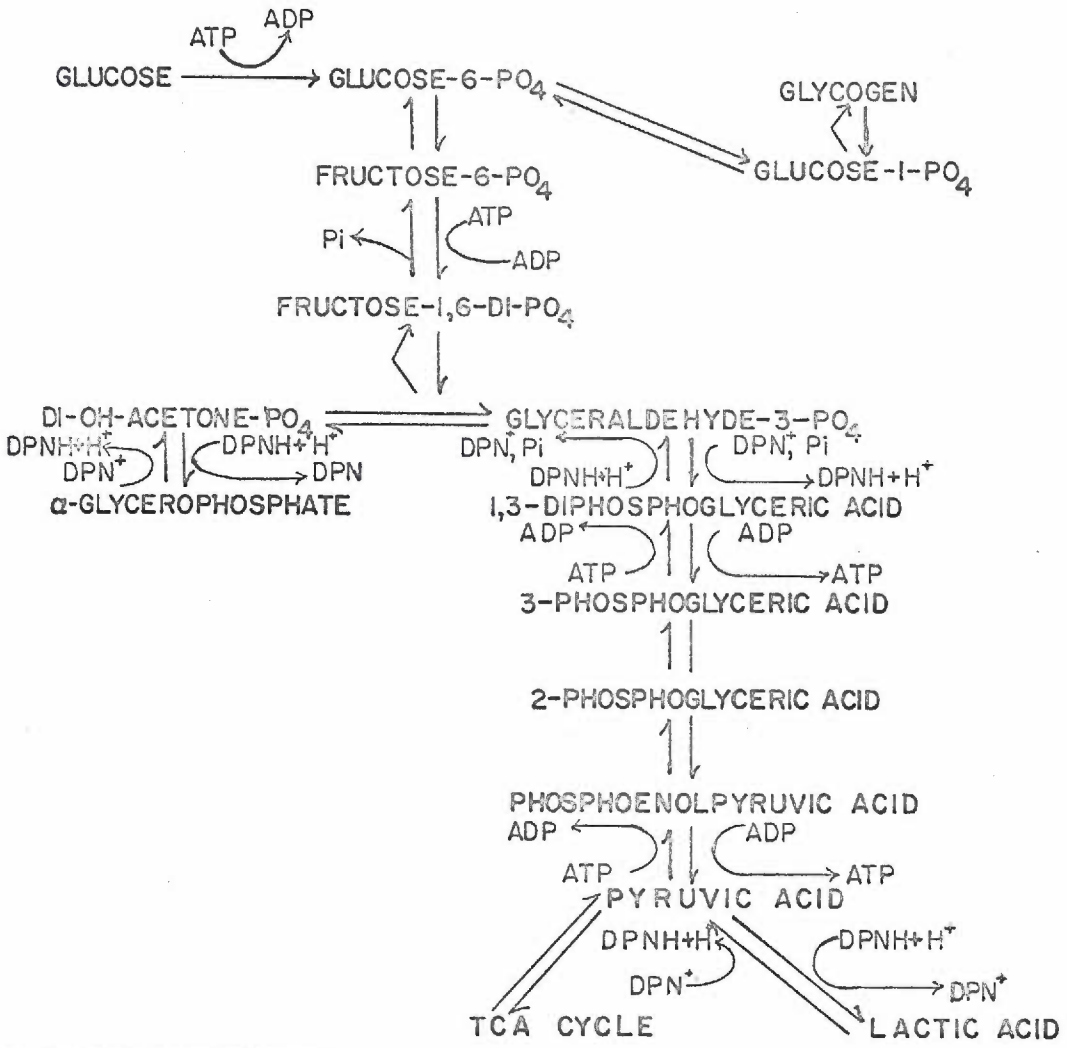
As indicated previously, by 1925 many basic differences in the chemistry, physiology and histology of muscle had been established. The comparative study of red and white muscle was relatively untouched for the next 25 years since the preoccupation in muscle metabolism studies seemed to be in the extraction and characterization of muscle proteins and purified enzyme systems. In more recent years, the interrelationship of these systems to the organized structure of the muscle cell has received much attention. An impetus to these studies has been given by the recently developed techniques of histochemistry and electron microscopy.

A. Muscle Histology

Figures III and IV indicate diagramatically the relation of the various muscle components to the muscle as a whole.

FIGURE 1

GLYCOLYTIC PATHWAY IN MUSCLE



Net production of ATP per 6-carbon unit

Anaerobically

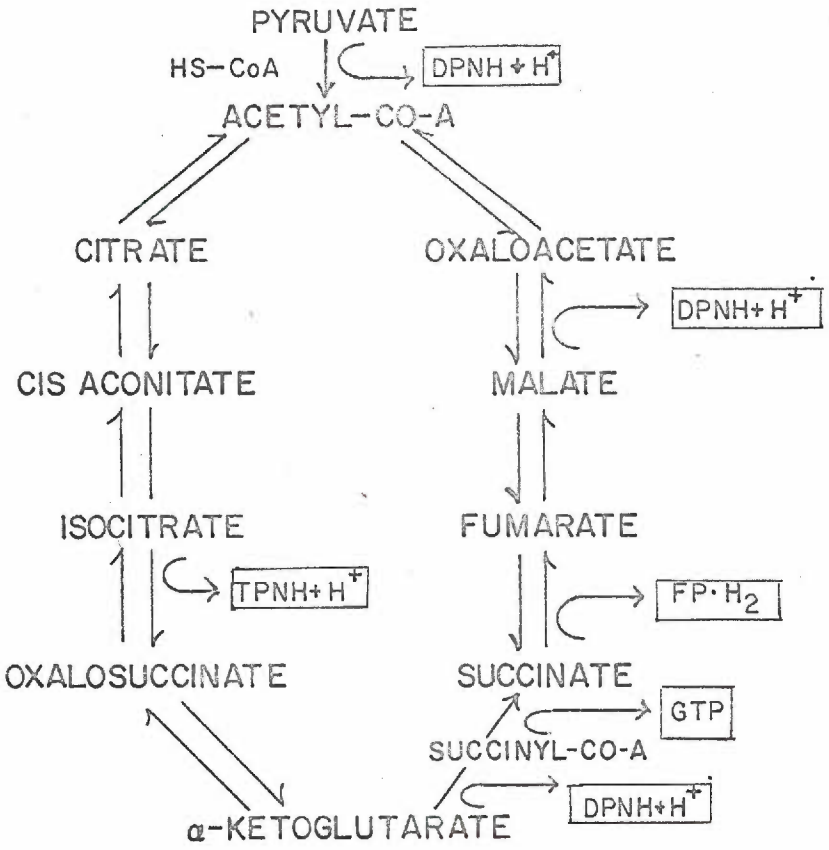
Glucose ⟶ fructose-1,6-di PO_4	-2 ATP
2 glyceraldehyde-PO_4 ⟶ 3-phosphoglyceric acid	+2 ATP
2 phosphoenol pyruvic acid ⟶ pyruvic acid	+2 ATP
Net ATP anaerobic conditions	+2 ATP

Aerobically

2 DPNH + H^+ ⟶ 2 DPN^+	+6 ATP
Net ATP aerobic conditions	+8 ATP

FIGURE 2

ENERGY YIELDING REACTIONS—CITRIC ACID CYCLE



Net production of ATP

1) Pyruvate + DPN^+ + HS-CoA ⟶ AcetylCoA + CO_2 + DPNH + H^+
   DPNH + H^+ ⟶ DPN^+ — -3 ATP

2) Isocitrate + TPN^+ ⟶ Oxalosuccinate + TPNH + H^+
   TPNH + H^+ ⟶ TPN^+ — -3 ATP

3) α-ketoglutarate + DPN^+ + HS-CoA ⟶ SuccinylCoA-CO_2 + DPNH + H^+
   DPNH + H^+ ⟶ DPN^+ — -3 ATP

   Succinyl CoA + GDP ⟶ GTP + Succinate
   GTP + ADP ⟶ GDP + ATP — -1 ATP

4) Succinate + Flavoprot. (FP) ⟶ $FP \cdot H_2$ + Fumarate
   $FP \cdot H_2$ ⟶ FP — -2 ATP

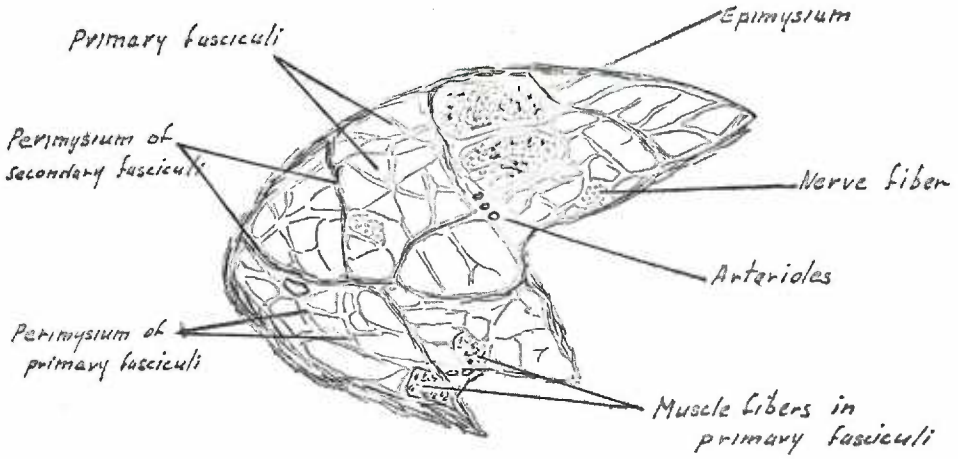
5) Malate + DPN^+ ⟶ Oxaloacetate + DPNH + H^+
   DPNH + H^+ ⟶ DPN^+ — -3 ATP

Net ATP per triose unit oxidized — -15 ATP
Net ATP per hexose unit oxidized — -30 ATP

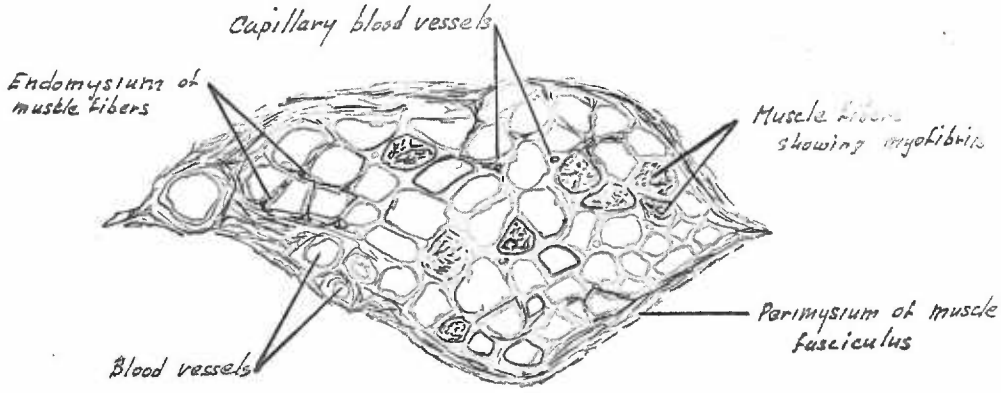
Figure 3 a. Schematic representation of a portion of a transverse section of an intact muscle indicating various gross structures of muscle.

Figure 3 b. Cross-section of a muscle fasciculus showing muscle fibers of a primary muscle fasciculus. Myofibrils are indicated in several of the fibers.

Figure 3



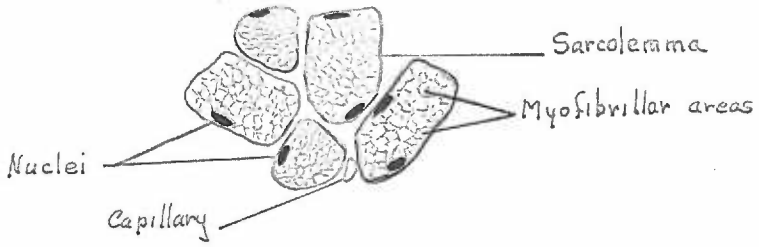
3a — Diagrammatic representation of transverse section of muscle



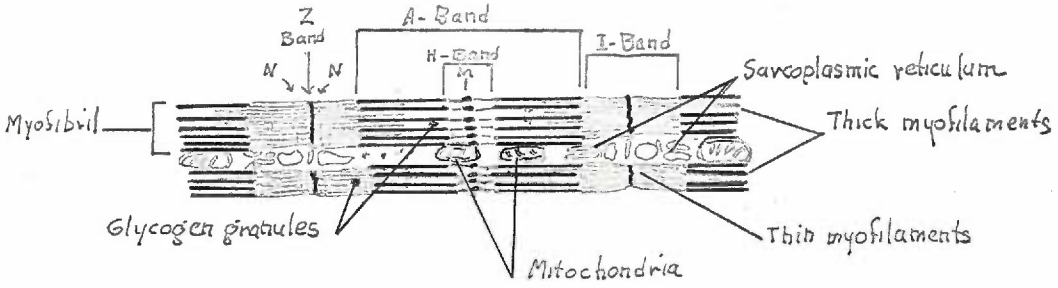
3b — Muscle fasciculus showing muscle fibers

Figure 4 a. A group of several muscle fibers enlarged from Figure 3 b. Interfibrillar network of sarcoplasmic reticulum is depicted, and the myofibrillar areas are indicated by the clear spaces within the fibers. Fibers of different sizes are represented.

Figure 4 b. Fine structure of muscle. Myofibrils are made up of alternating areas of thick and thin myofilaments. Sarcoplasmic reticulum, mitochondria and glycogen granules are indicated in relation to the A and I bands of the myofibrils.



4 a - Group of muscle fibers showing interfibrillar network



4 b - Schematic representation - fine structure of muscle

An intact muscle is surrounded by a fibrous connective tissue coating called the epimysium. This tissue extends into the interior of the muscle to the spaces between bundles of muscle as the perimysium. This stroma consists of irregularly arranged collagenous, reticular and elastic fibers, and a variety of connective tissue cells including fat cells. The thick perimysium which separates the muscle into muscle bundles or fasciculi, divides into fine strands and surrounds the individual fibers of a muscle fasciculus as the endomysium. The endomysium contains fibroblasts and fixed macrophages. Blood vessels and nerves are embedded in the perimysium, and capillaries extend into the endomysium[12].

The fiber (10 to 100 μ in diameter), the unit cell of muscle, is a multinucleated cell surrounded by a complex membrane, the sarcolemma. This consists of a basement membrane and a delicate network of associated reticular fibers. The sarcoplasm, the fluid matrix of the fiber, is a complex mixture of proteins which includes the glycolytic and other enzymes. Within the fiber are the myofibrils, the contractile units of the cell, arranged in band-like array, 1 to 2 μ in diameter, but often appearing flattened on cross-section. Longitudinally, the muscle fiber is characterized by alternating light and dark areas, giving the typical striated appearance. On electron microscopy these light (I band) and dark (A band) areas are due to the presence of thick and thin filaments which are composed of the proteins myosin and actin, respectively. Other bands of the myofibril have been identified: the Z membrane, the H zone, and the N band[49]. There is apparently no membrane separating the myofibrils and the sarcoplasm

which surrounds them. Located between the myofibrils are the mitochondria. In some muscles, the mitochondria are found opposite definite areas of the myofibrils, and in certain fibers a concentration of large mitochondria are found adjacent to the sarcolemma[90]. Within the fiber is found also a network of tubules and cisternae, the sarcoplasmic reticulum, which surrounds and interlaces the myofibrils and corresponds in some respects to the endoplasmic reticulum of other cells[99]. Located in the sarcoplasm are granular structures corresponding to glycogen granules, and also fat droplets. The amounts of mitochondria, sarcoplasmic reticulum, glycogen and fat varies with the particular type of muscle.

B. Electron Microscope Observations

Electron micrographs of muscle fibers have indicated marked differences between the two muscle types. Pellagrino and Franzini[97], using the soleus (red) and the medial head of the gastrocnemius (white) muscles of the rat, have found that the fibrils of the red fiber are more or less regularly rounded and separated by large sarcoplasmic spaces. The mitochondria are larger and more numerous than in the white fibers and are often located in large bundles at the periphery near the sarcolemma. The fibrils in the white muscle are less regular in shape and are separated by sarcoplasmic areas which are denser, narrower and less variable in width than in the red. The sarcoplasmic reticulum of both muscles shows two triad structures per sarcomere, although the cisternae of the sarcoplasmic reticulum are more extensively developed (in the gastrocnemius). Since white muscle is considered a more rapidly acting muscle than red,

the observation of a more highly developed sarcoplasmic reticulum is in keeping with the concept that this structure plays a role in the conduction of impulses into the fiber from the sarcolemma[48] and also in the mechanism of relaxation[8,80]. Glycogen granules were found to be evenly distributed in the white fibers, but appear to be arranged in clusters in the red[97]. These observations were made also in red and white fibers of the rat diaphragm[90] and rat sartorius and oblique muscles[99].

C. Metabolic Constituents

Several studies have confirmed that the concentration of myoglobin in red muscle is higher than in white. The content of myoglobin of a particular muscle can vary greatly from species to species. Lawrie[65] examined several species and reported variations in myoglobin content of the psoas muscle from 0.02% in the rabbit to 0.84% in the blue whale. Correlation between high oxidative activity, high cytochrome content and high myoglobin content have also been found[66, 45]. Paul and Sperling[93] have found a direct relation between the mitochondrial density and oxidative activity in red and white muscle from a variety of species. Ogata[88] has reported that in the rabbit homogenates of red muscle (soleus) have a QO_2 six times higher than homogenates of white muscle. He interpreted this to mean that red muscle oxidizes pyruvate and succinate at a rate about six times that of white muscle (posterior edge of the adductor magnus). Table I summarizes some of the differences found in relative concentrations of various metabolites in red and white mammalian muscle. Table II indicates some of the metabolic differences in red and white rat

TABLE I

Differences in Metabolic Constituents of Red and White Muscle of Various Mammalian Species as Cited in the Literature

Metabolite	Species	Muscle*		Concentration Ratio (w/r)	Reference
		Red	White		
Glycogen	Rabbit	S	A. mag	3.7	88
Glycogen	Rat	Sm	Sm	1.4	5
Glycogen[1]	Rat	Sm	Sm	0.74	5
Phosphocreatine	Rabbit	S	A. mag	1.5	88
Phosphocreatine[1]	Chicken Pigeon	L	P	2.0	50
Phosphocreatine[1]	Rat	Sm	Sm	1.3	5
Inorganic P	Rabbit	S	A. mag	2.0	88
ATP	Turkey	L	P	2.5	58
7″ ATP Hyd. P	Rabbit	S	A. mag	2.0	88
TASP	Rabbit	S	A. mag	1.5	88
Lactate production	Rabbit	S	A. mag	1.3	88
ATPase	Rabbit	S	A. mag	3.3	88
Anserine; Carnosine	Chicken	L	P	6.0	22
Lactic dehydrogenase	Guinea pig	-	-	2.7	11
α-glycerophosphate[2]	Rat	Sm	Sm	0.4	98
Cholesterol	-	-	-	1.3	28
Creatine-creatinine	Rabbit; Chicken	-	-	w > r	103
Creatine[1]	Rat	Sm	Sm	1.2	5
Pyrophosphate	Rabbit	S	-	1.5	69
CO_2 production	Rabbit	-	-	1.8	1
O_2 consumption	Rabbit	-	-	1.7	1

*S = soleus; A. mag = adductor magnus; Sm = semimembranosus; L = leg; P = pectoral; - = no special muscle listed.

[1]Warburg experiment, content in muscle after 2 hours incubation, Krebs bicarbonate buffered medium, pH 7.4.

[2]Warburg experiment, conditions as in 1, except incubated under hypoxic conditions (95% N_2 - 5% CO_2).

TABLE II

Metabolic Differences Between Red and White Muscle as Demonstrated in Warburg Experiments, Rat Adductor Muscle (The differences indicated are all statistically significant at the level of 1% or less.)

Metabolite	Concentration Red	Concentration White	Reference
	Krebs bicarbonate buffered medium[1]		
Acetoacetic acid uptake	0.53[2]	0.34	5
Lactate production	0.86[3]	1.10	5
α-glycerophosphate production (hypoxic)	0.06[4]	0.02	98
Glycine-1-C^{14} uptake[5]	43	59	6
	Glycylglycine buffered medium[1]		
Acetoacetic acid-3-C^{14} uptake	0.26[2]	0.19	5
Lactate (specific activity x 10^{-3})	52 ± 5	32 ± 5	5
CO_2 production (cpm/2 hr)	213	149	5
QO_2 (μl/mg hr): 1st hour	0.76	0.52	5
2nd hour	0.69	0.47	

[1]Plus 150 mg% glucose/100 ml medium, pH 7.4, 2 hours

[2]mg/g hr, wet weight

[3]mg/g, wet weight

[4]μmoles/g hr, wet weight

[5]cpm/mg protein in muscle fibers after 2 hours incubation

muscle previously demonstrated in Warburg experiments.

The values presented in Tables I and II agree with the general observation that white muscle contains a strongly developed glycolytic system and red muscle has a greater capacity for oxidative metabolism.

D. Histochemical Observations

On histochemical examination, red skeletal muscle, in general, has been found to consist predominantly of small fibers, rich in oxidative enzymes, whereas in white muscle the majority of the fibers are larger in size and poorer in oxidative enzymes[113]. However, variation in enzyme content without variation of fiber size has been reported[82]. Fibers of intermediate size and enzyme activity have also been described[113]. Differences in oxidative enzyme content (succinic dehydrogenase, cytochrome oxidase) of muscle fibers has been shown histochemically to occur in many different species including the pigeon[34], rat, mouse, guinea pig and rabbit[108], rat and pigeon[26], bat[31], rat[89], cat[87] and human[24]. In addition, using histochemical techniques, red muscle fibers have been found to have a higher lipase activity[32], β-OH butyrate dehydrogenase[24], TPNH, DPNH diaphorases (dehydrogenase)[108] and mitochondrial α-glycerophosphate dehydrogenase (α-GPDH) activity[44]. Histochemically, white fibers have been shown to have higher phosphorylase[25,26] aldolase DPN-linked α-GPDH[35] and glyceraldehyde-3-PO_4 dehydrogenase (44) activities. Previous histochemical evidence has indicated that lactic dehydrogenase[24] and α-GPDH[94] are more active in red than in white muscle. However, Van Wijhe et al[47] found that these glycolytic enzymes are more active in white muscle provided phenozine methosulfate

is present as electron carrier. In addition, Blanchaer and Van Wijhe[10] have found that red muscle contains all five isozymes of lactate dehydrogenase, whereas white contains only isozymes IV and V (the muscle or M type), which are active at pyruvate concentrations that are inhibitory to Type I (heart or H type)[75]. Dubowitz and Pearse[25,26] have observed a reciprocal relationship between phosphorylase content and oxidative enzymes in human skeletal muscle.

The observation by Hess and Pearse that transglucosylase activity is more active in red fibers than in white[42] was surprising since it is generally agreed that the metabolic pathways are different for glycogen synthesis and glycogen breakdown. The apparent dissociation of glycogen synthetic and catabolic activity could not be explained. Since this evidence is based on histochemical procedures, the possibility exists that a metabolic block may have been present in the histochemical method as was demonstrated in the case of lactic dehydrogenase[122].

It is interesting to note that at the subcellular level phosphorylase and transglucosylase are associated with different components of the muscle cell. Phosphorylase is one of the soluble enzymes of the sarcoplasm. Transglucosylase activity, however, is associated with the fraction of muscle homogenates corresponding to the microsomes or fragmented sarcoplasmic reticulum[104,2].

Since there is no data published concerning the quantitation of phosphorylase and transglucoslyase activities in red and white muscle of rats, these activities were determined as a portion of the investigation of glycogen metabolism in muscle described in this paper.

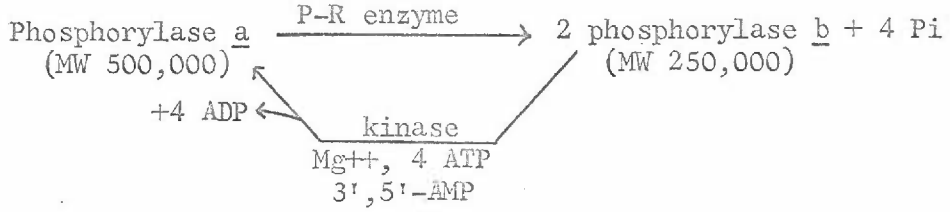
V. Enzymatic Relations in Glycogen Synthesis and Breakdown

A. Phosphorylase

Until 1957 glycogen synthesis and breakdown was attributed to a reversible reaction mediated by the enzyme phosphorylase.

$$\text{glucose-1-PO}_4 + \text{glycogen primer} \rightleftharpoons \text{glycogen-1-glycosyl} + \text{Pi}$$

Phosphorylase was characterized by the Cori group in a series of studies which were initiated in the observations of the relation of glucose-1-PO_4 to glycogen breakdown and formation of blood glucose. The Cori group isolated and characterized muscle phosphorylase[38]; they found that the muscle enzyme exists in an inactive (b) and active (a) form, and that adenosine monophosphate (AMP) is required for enzymatic conversion of the inactive to the active form[17] in muscle. The relationships of the interconversion of the two forms in muscle are indicated in the following diagram.



Muscle phosphorylase a, molecular weight 500,000, is split into two monomer units by the action of phosphorylase rupturing enzyme (P-R enzyme) or phosphate removing enzyme. Phosphorylase b is considered to be a dimer, each unit of which contains one binding site for PO_4, probably phosphoryl serine[46]. Conversion of the b to a form is mediated by phosphoryl kinase[55], which in turn is activated by

cyclic adenosine phosphate (3',5'-AMP). The action of epinephrine in increasing glycogenolysis may be at the level of adenyl cyclase, an enzyme capable of forming 3',5'-AMP from ATP[100]. The increase in cyclic AMP in turn activates the phosphorylase kinase to convert increased amounts of the b to the a form. Danforth et al[54] found that levels of phosphorylase a rise from resting levels of 5% to 100% of the total phosphorylase in three seconds after onset of a tetanic contraction, but conversion by action of epinephrine requires periods of time in terms of minutes for a maximum of 55% increase. Dichloroisoproterenol (an analog of epinephrine) blocks the epinephrine effect, but has no effect on the increase in phosphorylase a produced by muscle work. It was suggested that the activity of phosphorylase b kinase is rate limiting in regulating muscle levels of phosphorylase a rather than P-R enzyme activity. The in vitro demonstration of the b to a conversion in muscle has been shown to be dependent on the presence of AMP. Helmrich and Cori[41] have suggested on the basis of kinetic studies that AMP induces conformational changes in the enzyme which result in an increased affinity for inorganic phosphate and glycogen.

Since the anabolic action of phosphorylase produces a straight chain or amylose type of polysaccharide, the synthesis of highly branched glycogen results from the combined action of phosphorylase and amylo- (1-4 → 1-6)-transglucosidase or branching enzyme[61]. Also, the catabolic action of phosphorylase on glycogen proceeds with the liberation of glucose-1-PO_4 units from the outer tiers of the molecule and stops as a branching point is ~~reached~~ approached. The residual glycogen

is termed a limit dextrin. The debranching enzyme or amylo-1,6,glucosidase removes the glucose units attached by the 1,6 linkage at the branching points and exposes a second layer of amylose chains to the action of phosphorylase[56].

Although glycogen synthesis occurring via the phosphorylase enzyme has been well established *in vitro*, certain observations began to cast doubts on the assumption that this reaction takes place to an appreciable extent *in vivo*.

1. In *in vitro* systems the ratio of Pi/glucose-1-PO_4 was found to govern the direction of phosphorylase activity. If this ratio was greater than 3, glycogenolysis predominated, and if the ratio was less than 3, glycogenesis resulted. However, Larner and Villar-Palasi[57] found *in vivo* that glycogen synthesis occurs with ratios as high as 305.

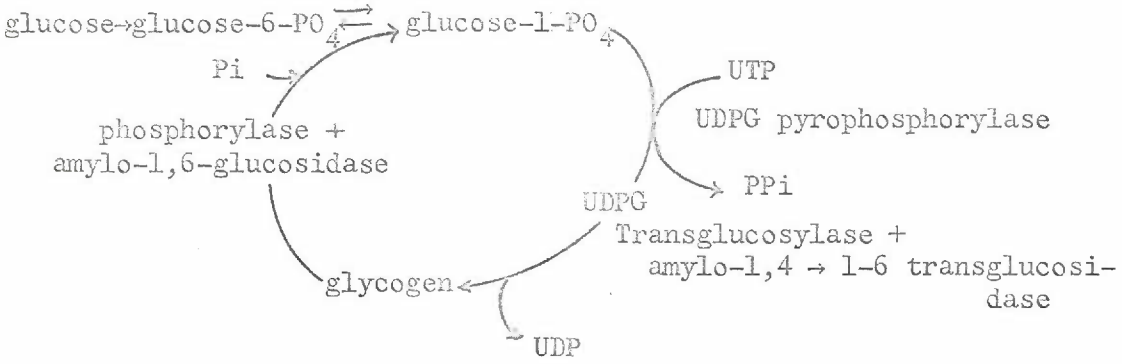
2. Cori and Illingworth[18] noted that under conditions in which active phosphorylase is increased, the higher enzyme activity is reflected only in the direction of increased glycogenolysis, and never in a higher rate of glycogenesis.

3. Certain observations made in studying glycogen storage diseases in humans indicate that abnormally high muscle glycogens can be found even when the phosphorylase levels are low[63,111], implying that glycogen synthesis occurs in the presence of low phosphorylase levels.

B. Uridine Diphosphoglucose-α-glucan Transglucosylase

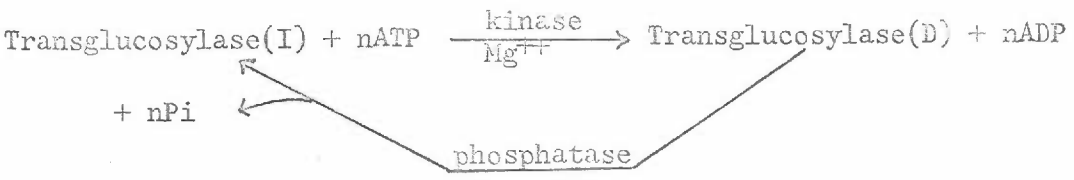
Niemeyer in 1955[85] suggested that glycogen synthesis may proceed via uridine diphosphoglucose (UDPG). However, it was Leloir and

Cardini[63] in 1957 who first showed that the glycogen content of an aqueous extract of liver increases in the presence of UDPG. Robbins et al[64] found that glycogen synthesis could take place from UDPG in homogenates from pigeon breast muscle. Villar-Palasi and Larner in 1960[123] proposed a cyclic nature for glycogen metabolism in which uridine diphosphoglucose-α-glucan transglucosylase (transglucosylase) causes glycogen formation and phosphorylase acts only in glycogen breakdown. The following diagram outlines the reactions involved in glycogen metabolism in muscle.



This mechanism shows that UDPG is the immediate precursor of glycogen and that UDPG is formed from glucose-1-PO_4 in an exchange reaction with uridine triphosphate (UTP). UDPG-α-glucan transglucosylase transfers glucosyl residues to lengthen the amylose 1-4 chain units and, under the influence of the branching enzyme (amylo-1,4 1,6-glucosidase) fragments of the terminal chains are removed and the 1,4 linkages altered to 1,6 bonds to become branch points on either the original amylose chains or at other sites on the glycogen molecule more centrally located.

Recent studies of Friedman and Larner[30] have indicated that transglucosylase exists in two forms, a glucose-6-PO_4 dependent (D) or inactive form and a glucose-6-PO_4 independent (I) or active form. Conversion of the inactive to active form is dependent on the presence of glucose-6-PO_4 as cofactor. Insulin, in the absence of glucose-6-PO_4, is able to convert the D to the I form. Friedman and Larner have proposed that the interconversions of transglucosylase may follow the reactions:



On comparing this activation-inactivation relationship with that indicated for phosphorylase (page 20), it is seen that the mechanisms are similar, yet opposite in effect. That is, a dephosphorylation of the D transglucosylase yields the I or more active form of the enzyme, whereas in phosphorylase, phosphorylation produces the active form. Since phosphorylase and transglucosylase exert opposing effects on glycogen metabolism, and since it appears that kinase activities can increase the relative activity of the catabolic enzyme and decrease the activity of the anabolic enzyme, Friedman and Larner suggest that both enzymes could be subject to simultaneous regulation through control of a common kinase (or phosphatase), or that control could be effected through a mechanism which could act in an identical manner on two separate but similar kinases (or phosphatases). One such common

control mechanism may be cyclic AMP, since Belocopitow[7] has found that this nucleotide inhibits transglucosylase but activates phosphorylase. An inhibition of phosphorylase by UDPG has also been reported[72]. Also, Appleman et al[3] suggest that Ca^{++} may be involved in a regulatory role, as this ion stimulates the transglucosylase I to D conversion and has been indicated as involved in the stimulation of phosphorylase b kinase. This is an interesting observation since recent theories of mechanism of muscle contraction suggest that on stimulation Ca^{++} is released from the sarcoplasmic reticulum and acts to form a link between the ADP of actin and the ATP of myosin cross bridges, forming actomyosin, which leads to subsequent contraction of the muscle protein. Contraction takes place as a result of the sliding action of interdigitating thick and thin myosin and actin filaments as long as the Ca^{++} is present. Ca^{++} is removed by action of the relaxing factor through an unknown mechanism and is "pumped" back into the sarcoplasmic reticulum[23]. If these theories are correct, the act of stimulation of muscle causes muscle contraction as well as stimulates glycogenolysis and inhibits glycogenesis. A mechanism coupled to muscle activity may explain the rapid conversion of phosphorylase b to a observed on tetanic stimulation by Danforth et al[21]. Another pertinent observation was that phosphofructokinase (PFK) is associated with the sarcoplasmic reticulum[74]. Mansour and Mansour[73] demonstrated that in the liver fluke PFK activity is increased in the presence of Ca^{++} and cyclic AMP. Since PFK is rate-limiting in the glycolytic pathway[84], increased activity of this enzyme mediated by Ca^{++} or cyclic AMP activation would fulfill the need for increased

glycolytic activity in muscle contraction and may be complemented by increased glycogen breakdown.

VI. Aims of Experimental Work Presented in This Thesis

Although it is now generally accepted that glycogen synthesis and degradation are mediated by two different metabolic pathways, literature references are found to an apparent dissociation of the activities of the two enzymes which are supposedly intimately bound by function to a common locus in the cell.

1. As previously mentioned, Hess and Pearse have reported dissociation of phosphorylase and transglucosylase in red and white muscle fibers in the rat[42]; that is, the red fibers have a higher transglucosylase activity and white fibers a higher phosphorylase activity.

2. Bo[13], using histochemical techniques, was able to detect phosphorylase activity in uterine smooth muscle, but no transglucosylase activity. These histochemical results were verified on homogenates of rat uterine muscle, also by Bo[14], who used the standard method for transglucosylase activity of Leloir and Goldemberg.

3. Takeuchi[120] failed to demonstrate transglucosylase activity in leukocytes, although phosphorylase activity and glycogen were present. However, Miller[79], using fresh cell preparations did find transglucosylase activity in white cells.

4. There is also the statement by Pearse[95] that neither phosphorylase nor transglucosylase are found in the epidermis of common laboratory animals and his suggestion that therefore carbohydrate metabolism must be carried out by some alternate pathway.

5. In contradiction to the results of Hess and Pearse[42], Engel[29], using histochemical techniques, found that in human muscle both transglucosylase and phosphorylase activities are high in the pale, large fibers.

In view of these observations, it seemed that further investigation of the relation between glycogen, transglucoslyase and phosphorylase should be carried out. The first step was to test the histochemical observation that white muscle fibers contain more phosphorylase and less transglucosylase activity than do the red muscle fibers. This was done by assaying phosphorylase and transglucosylase activities in predominantly red and predominantly white muscle fiber groups in the rat. Such muscle fiber groups have been found suitable for metabolic experiments[4].

A second series of experiments investigated the in vitro incorporation of glucose-C^{14} into the glycogen fractions of both types of muscle. Because of the present controversy over the metabolic significance in tissues of glycogen soluble in trichloracetic acid and of residual glycogen[106,40] it was decided to fractionate the glycogen and observe any differences in incorporation of C^{14} into the fractions. Viability of the tissues was checked by determining the $C^{14}O_2$ production over the experimental period. It was not possible to determine oxygen consumption since a bicarbonate buffered solution was used for the incubation medium.

The in vitro results were checked by a series of in vivo experiments in which the incorporation of U-C^{14} glucose into glycogen was determined after intravenous injection of the isotope. The relative

amounts of red and white fibers in the *in vitro* and *in vivo* experiments were checked by histochemical determination of succinic dehydrogenase, and in later experiments by quantitative determination of succinic dehydrogenase in muscle homogenates.

Using this experimental approach, it was hoped that further information on the metabolic differences between red and white muscle would be found.

MATERIALS AND METHODS

I. Animals and Maintenance

The animals used in these studies were female Sprague Dawley rats, weighing from 220 to 270 grams, and maintained in a constant temperature room at 26-27°C on Purina chow.

II. Phosphorylase

A. Tissue Sampling

Fed rats were lightly anesthetized with Nembutal (3.5-4 mg/100 g body weight) to decrease muscle spasms, decapitated, and exsanguinated. The skin and outer muscles of the hind limbs were dissected away exposing the semimembranosus muscle. A portion of this muscle, near its insertion in the tibia, was grasped with fine pointed forceps and dissected from the rest of the muscle with a sharp pointed scalpel (blade No. 11). With the muscle still attached at the proximal end, the muscle was divided into fiber groups approximately 3-4 mm in thickness by inserting the scalpel in the muscle and gently slicing down the length of the muscle. Three to four such divisions were made depending on the thickness of the section of muscle dissected free. In the exsanguinated rat it was possible to differentiate the color of the muscle fibers and only the most pale and the deepest red were selected; the intermediate-colored fibers were discarded. The muscle was then cut free at the proximal end. The outer fibers were lightest in color, and the innermost fibers darkest. Approximately 100-150 mg of tissue was quick-frozen in tubes resting in crushed dry ice. By placing the fibers in contact with the walls of the tubes, freezing was extremely rapid. A pair of red and white muscle samples

was obtained from each hind leg and frozen within ten minutes after decapitation.

B. Tissue Preparation and Homogenization

The frozen tissues were transferred to previously tared homogenizing tubes (Kontes, Size B, conical tip) and weighed. The tissue was brought to the upper area of the tube, moistened with 0.1 ml of 0.15 M potassium fluoride (KF) or 0.15 M KF in 0.001 M ethylene diaminetetraacetic acid (EDTA), minced with a scalpel, and refrozen in dry ice. This procedure facilitated the homogenizing process. The muscles were homogenized in an amount of 0.15 M KF or 0.001 M EDTA in 0.15 M KF solution to make a 10% tissue weight-to-volume suspension. The homogenizing tubes were held in an ice bath and the tissue ground for 30-40 seconds at high speed (Con-torque Power Unit, Eberbach Corp.), raising and lowering the tube on the pestle about 10 times. The tubes were checked for pieces of tissue lodged in the tip of the tube. These pieces were raised to the side of the tube with a glass rod and the tissue homogenized for an additional 20-30 seconds. Total homogenization time was always less than one minute. It was possible generally to distinguish homogenates of red and white muscle by the color of the homogenate, the white muscle preparation being pale in comparison to the reddish color of the red muscle homogenate.

In some assays, an aliquot of the whole homogenate was used. However, in the majority of assays, 0.8 to 1.0 ml aliquots of well-mixed homogenate were pipetted into small tubes (10 mm x 75 mm), covered with parafilm and centrifuged (825 x g for 10 minutes at

0-2°C). Phosphorylase activity of the supernatant was determined.

C. Assay Procedure

The phosphorylase method was essentially that developed by Cori, Illingworth and Keller[19] as modified for tissue homogenates by Leloir and Goldemberg[68], and Niemeyer et al[86]. Phosphorylase activity was measured in the direction of glycogen synthesis by determining the amount of inorganic phosphate liberated when the glucose moiety of glucose-1-phosphate was transferred to glycogen. The overall reaction may be indicated by:

$$x \text{ glucose-1-PO}_4 + (\text{glycogen})_n \rightleftarrows (\text{glycogen})_{n+x} + x \text{ inorganic phosphate}$$

Total phosphorylase was determined in the presence of the activator adenosine monophosphate (AMP) which, in vitro, is necessary for the conversion of inactive phosphorylase b to the active a form. The enzyme which reacts in vitro in the absence of AMP is termed active phosphorylase. Because the amount of active phosphorylase is dependent on the activity of the enzymes mediating a to b conversion, the phosphorylase rupturing enzyme (P-R enzyme), the homogenizing media contained EDTA and fluoride which are known to inhibit the P-R enzyme and phosphorylase kinase[21]. Since a crude extract was used, the reaction was carried out at pH 6.0 to minimize phosphoglucomutase activity which might remove glucose-1-PO_4 from the reaction mixture.

1. Reagents

a. Homogenizing media: 0.001 M ethylenediaminetetraacetic acid (EDTA) in 0.15 potassium fluoride; stored at 4°C.

b. Citrate buffer: 0.15 M, pH 6.0; stored at 4°C.

c. 5% glycogen in 0.75 M potassium fluoride; stored at 4°C. The glycogen was an Eastman product, prepared from oysters.

d. 0.075 M cysteine (cysteine hydrochloride-monohydrate, California Biochemicals Corporation). This solution was made fresh daily and the pH adjusted to 6.0 with alkali immediately before use. Five ml of this solution was diluted with an equal volume of 0.15 M citrate buffer, pH 6.0, and stored in crushed ice before use.

e. Substrate A: 80 mM glucose-1-PO_4 (dipotassium salt with 2 H_2O, A grade, assay 99.2%, California Biochemicals Corporation).

Substrate B: 80 mM glucose-1-PO_4 plus 5 mM adenosine monophosphate (AMP) sodium salt (Sigma type III). The pH of these substrates was adjusted to 6.0; 0.9 ml aliquots were stored at -18 to -20°C.

2. Method

During centrifugation of the homogenates, the following reaction mixture was prepared and stored in crushed ice:

0.4 ml citrate buffer-cysteine solution;

0.2 ml 0.5% glycogen-0.75 M KF;

0.2 ml Substrate A or Substrate B.

With the addition of 0.2 ml of the diluted samples, the final volume of the reaction mixture was 1 ml. The diluted

samples were prepared by taking a 0.5 ml aliquot of the whole homogenate or supernatant and diluting 1:11 with the 0.001 M EDTA in 0.15 M KF. A one ml aliquot of this diluted solution was then further diluted 1:2 with the citrate buffer-cysteine solution. The above reaction mixtures were preincubated for 10 minutes at 37°C. Subsequent incubations were also carried out at this temperature. Two-tenths ml of the diluted homogenate or supernatant was added, the tube shaken and a 0.2 ml aliquot withdrawn immediately (zero time sample) and pipetted into 0.5 ml 8% trichloracetic acid (TCA) to terminate the reaction. The zero time sample tube was stored in an ice bath as the acid hydrolysis of the substrate is accelerated at room temperature. Two-tenths ml aliquots were withdrawn from the incubation mixture at 8 and 16 minute intervals. Four muscle samples (two red and two white) were run simultaneously at one minute intervals.

3. Phosphate determination

The colorimetric procedure was a modification of the method of Gomori[37].

a. Reagents

(1) Molybdic-sulfuric reagent: 2 volumes of a 5% solution of sodium molybdate ($Na_2MoO_4.2H_2O$), 1 volume of 10 N sulfuric acid and 1 volume distilled water.

(2) Reducing solution: 1 g Elon (p-methylamino-phenol sulfate) in 100 ml of a 3% solution

of sodium bisulfite.

(3) The above two reagents were diluted 1:2 with distilled water for use.

b. Method

To the tubes containing the aliquots of incubation media plus TCA were added 2 ml of the molybdic-sulfuric reagent and 0.4 ml of the Elon, making a final volume of 3.1 ml. The tubes were mixed well, allowed to stand for 30 minutes for color development, centrifuged 10 minutes at 3000 rpm at 20°C and read at 690 mμ in the Beckman DU spectrophotometer.

A calibration curve for this determination was made for concentrations of phosphorus ranging from 1-20μg.

c. Results were expressed as μg P per gm tissue, wet weight, per minute.

III. Uridine diphosphate α-glucan transglucosylase activity

A. Tissue Sampling and Homogenization

Muscle samples were obtained from fed rats as previously described (IIA) with the exception that in addition to the semimembranosus muscle, red and white fiber groups were obtained from the semitendinosus and from the white muscle area of the gastrocnemius and red muscle of the soleus. Three pairs of red and white muscle were obtained from each rat and stored at dry ice temperature.

Tissues were weighed and a 10% w/v homogenate made as previously described with the exception that 0.25 M sucrose in 0.001 M EDTA was

was used as the homogenizing medium. The homogenates were either stored in crushed ice to be assayed within an hour or stored for longer periods at dry ice temperature. Five-tenth ml aliquots of the homogenate were placed in 7 x 50 mm tubes and centrifuged five minutes at 600 g at 0-2°C.

B. Assay

The assay procedure for UDPG-glycogen transglucosylase activity was essentially that of Leloir and Goldemberg[68], and was carried out in two enzymatic steps: (1) In the presence of added UDPG and glucose-6-PO_4, transglucosylase of the tissue transfers glucose from UDPG to glycogen, forming uridine diphosphate (UDP)

$$(\text{glucose})_n + \text{UDPG} \rightarrow (\text{glucose})_{n+1} + \text{UDP}.$$

(2) The UDP formed is reacted with phosphoenol pyruvic acid in the presence of pyruvate kinase

$$\text{UDP} + \text{PEP} \xrightarrow{\text{kinase}} \text{UTP} + \text{pyruvic acid}.$$

The amount of pyruvic acid produced is determined by formation of the 2,4,dinitrophenylhydrazone which is quantitated colorimetrically.

1. Reagents. Step I:

   a. Homogenizing media: 0.25 M sucrose in 0.001 M EDTA, pH adjusted to 8.5; stored at 4°C.

   b. 30 μmoles/ml uridine diphosphate glucose--sodium salt (Sigma, 90-100% assay); 0.45 ml aliquots stored at -20°C.

   c. 60 μmoles/ml glucose-6-phosphate--disodium salt (Sigma, 100% assay); 1 ml aliquots stored at -20°C.

d. 0.9 mmoles/ml glycylglycine buffer, pH 8.5; stored at 4°C.

e. 4.8 mg glycogen/ml in 30 mM EDTA; stored at 4°C.

Five-hundredths ml each of the UDPG, glucose-6-PO_4 and buffer, and 0.1 ml of the glycogen-EDTA solution were combined in the reaction mixture. Final volume was 0.3 ml with addition of 0.05 ml of the homogenate supernatant.

Final concentrations of the above reagents in the reaction mixture were: glycogen, 0.8%; UDPG, 5 mM; glucose-6-PO_4, 10 mM; glycylglycine buffer, 0.15 M; and EDTA, 5 mM.

Reagents. Step II:

a. Pyruvate kinase (muscle), Nutritional Biochemicals Corporation, ammonium sulfate suspension, diluted 1:20 with 0.075 M magnesium sulfate; 0.9 ml aliquots stored at -20°C.

b. 7.5 mM phosphoenol pyruvic acid ($Na_3.4-1/2H_2O$), California Biochemicals Corporation, dissolved in 0.3 M KCl; 1 ml aliquots stored at -20°C.

c. 0.1% dinitrophenylhydrazine in 2 N HCl.

d. 10 N NaOH.

e. 95% ethanol.

f. Uridine diphosphate standards (California Biochemicals Corporation). Stock solution, 10.19 µmoles/ml; 0.5 ml aliquots stored at -20°C.

2. Method

In the initial transglucosylase assays, a run consisted of duplicate blanks containing 0.05 ml of sucrose-EDTA solution instead of homogenate, duplicates of two concentrations of standards (0.25 and 0.5 μmole/0.05 ml UDP) and duplicate aliquots of a red and white muscle homogenate. The reaction mixtures for Step I were preincubated for 10 minutes at 37°C, 0.05 ml of the supernatant or whole homogenate added, and a 0.05 ml aliquot withdrawn for the zero time sample at 37°C. Additional 0.05 ml aliquots were withdrawn at five and 10 minute intervals. When it was determined that the method was highly reproducible (see section on results), the incubation volume was changed in later experiments to 0.2 ml and the concentration of the reagents adjusted to give the final concentrations indicated above. By withdrawing samples at zero time and after twelve minutes incubation, six muscle samples were determined with the blanks and standards in a single run.

The 0.05 ml aliquots from the blanks, standards and muscle samples were pipetted into tubes and heated in a boiling water bath for 2 minutes to terminate the enzyme reaction. The tubes were placed in crushed ice and after the final sample had been withdrawn, 0.05 ml phosphoenol pyruvate and 0.05 ml pyruvate kinase solutions added to all tubes, samples, blanks and standards. The tubes were incubated for 15 minutes at 37°C, the reaction terminated by the

addition of 0.3 ml of dinitrophenylhydrazine reagent and then allowed to stand 5 minutes. Four-tenths ml of 10 N NaOH was added to each tube, the tubes shaken to mix thoroughly, and then 2.2 ml 95% ethanol added. The tubes were placed on a Vortex mixer for a few seconds, allowed to stand for 10 minutes, centrifuged for five minutes at 3000 rpm and read at 520 mμ in the Beckman DU. Results are expressed as μmoles of UDP formed per gram of tissue, wet weight, per minute.

IV. In vitro Warburg Studies

The Warburg flasks used (22-25 ml volume, double side arms) contained 2 ml of Krebs bicarbonate buffer, pH 7.4, plus 150 mg/100 ml glucose. This medium was made according to directions given in Umbreit et al[121] with the exception that the concentration of $CaCl_2$ was approximately one-third and that of $MgSO_4$ one-half of that listed in the directions. The formation of insoluble salts in the medium was prevented by using these lower concentrations. Five-tenths ml of the medium containing 2.5μc of uniformly labeled glucose-C^{14} (UL) (Volk, 0.1 mc/1.5 mg) was pipetted into one side arm fitted with a conventional venting plug. The other side arm was closed with a rubber vial closure and secured with a rubber band.

A. Tissue Sampling

Rats were anesthetized with a light dose of Nembutal (2.5 mg/100 g body weight) and decapitated. The red and white muscle fiber groups were dissected from the semimembranosus and red muscle fiber groups from the caudofemoralis of the hind legs as previously

described (IIA). The fiber groups were approximately 2 mm thick and 25-30 mm in length. During the dissection procedure, the fibers were placed in Krebs bicarbonate buffered medium, pH 7.4, at room temperature, and a stream of 95% O_2 + 5% CO_2 bubbled through the medium to insure adequate oxygenation for the fibers. Muscle tissue from two animals were combined for one experiment.

B. Warburg Procedure

An experimental run consisted of 16 flasks, eight each for the red and white muscle samples. The tissues were drained of excess fluid on filter paper, placed into previously weighed Warburg flasks and flushed with 95% O_2 + 5% CO_2 for one minute. The flasks were reweighed, attached to the manometers, and placed in the water bath at 37°C. In the first experiment, muscle samples weighing 15 to 50 mg were used. In later experiments, the amount of tissue was increased and averaged from 55 to 103 mg per flask. All flasks were flushed with the oxygen-carbon dioxide mixture for five minutes by means of the gassing manifold of the Warburg apparatus. After an equilibration period of 15 minutes, the glucose-C^{14} substrate from the side arm was rinsed into the main chamber of the flasks to begin the experimental period.

At the end of the equilibration period, 0.1 ml of 18 N H_2SO_4 was added from a 1 ml syringe directly to the medium of a pair of flasks containing the red and white muscle by inserting the needle (22 G, 2 inch) through the vial closure into the large chamber of the flask. These tissues provided the control values for the glycogen content of the muscle.

At 10, 20, 30, 45, 60, 90, and 120 minute intervals, a pair of flasks, containing either red or white muscle, was removed from the incubating bath, 0.4 ml of 9.5 N NaOH added to the side arms and 0.1 ml of 18 N H_2SO_4 added to the medium through the vial closure. The acid terminated the experimental period and the CO_2 liberated by acidification of the medium was trapped in the alkali. The flasks were returned to the water bath and shaken for an additional 40 minutes to insure maximal absorption of the $C^{14}O_2$ from the gas phase.

C. $C^{14}O_2$ Collection and Assay

The flasks were detached from the manometer, the vial closures removed and the NaOH withdrawn with a capillary pipette and placed in 4- or 5-ml volumetric flasks. The side arms were rinsed with several aliquots of distilled water, the washings added to volumetric flasks, and the contents made to volume. The radioactivity was determined by plating triplicate 0.2 ml aliquots on 1-1/4 x 3/32 inch stainless steel planchets. Disks of lens paper (1-1/8 inch diameter) placed in the planchets allowed even spreading of the sample. One-tenth ml of an alkaline glue solution was added to insure that the lens paper adhered to the planchet. The alkaline glue solution consisted of 95 ml of a 5% solution of glucose in 20% ethanol plus 5 ml of 1% aerosol and 1 ml of 0.4 N NaOH. The samples were dried at 80°C for one-half hour and counted in a Tracerlab Omniguard low background instrument.

D. Criteria for Acceptable Levels of Radioactivity in Samples

Because the C^{14} activity in a few samples was low, the following criteria were observed in accepting a count as significant.

(a) A minimum of 1600 total counts was collected in order to insure that the percent of error of the observed count was statistically significant at the 1% level. An acceptable count had to be 1 count per minute above the range of the mean background count $\pm$ 3 x SD and amounted to a minimum of 3 cpm.

E. Determination of Tissue Glycogen

The tissue was removed from the flasks and thoroughly rinsed in two 100-ml quantities of distilled water. The tissue was then drained, placed in small tubes and stored in the deep freeze. These samples were later thawed and homogenized in 1 ml of 10% trichloracetic acid (TCA) in round-bottomed all-glass homogenizers of the Potter Eljehem type by means of the motor unit previously described. Care was taken to keep the grinding surface submerged in an ice bath to minimize the effects of friction heat. The pestle was rinsed with 0.5 ml of 10% TCA and the contents of the tube mixed on a Vortex mixer and allowed to stand at room temperature for one hour.

1. Glycogen Fractionation

The partition of glycogen into TCA and residual fractions was adapted from the method of Russel and Bloom[110]. All centrifugations were carried out at 3000 rpm at 2-4°C. The homogenizing tubes containing the ground tissue in 1.5 ml TCA were centrifuged 15 minutes and the supernatant decanted into 10 ml conical centrifuge tubes. The precipitated tissue was resuspended in 0.5 ml 10% TCA, mixed on the Vortex, recentrifuged 15 minutes, and the supernatants combined. The TCA extract was stored in the deep freeze

at -20°C.

a. Residual glycogen

The precipitated tissue was washed by suspending in 1 ml 95% ethanol (ETOH), mixed on the Vortex, and recentrifuged for five minutes. Five-tenths ml of 30% KOH was then pipetted into the tubes and the precipitate digested in a boiling water bath for 15 minutes with frequent shaking. The tubes were cooled and stored in the deep freeze (-20°C).

On the day of analysis, 1 ml of absolute methanol was added to the KOH-digested samples, the contents of the tubes were mixed well, and the tubes placed in a boiling water bath until the contents began to boil. This process hastened the precipitation of glycogen. The tubes were allowed to stand at room temperature for four hours when the precipitated glycogen was recovered by centrifuging 15 minutes. The supernatant was discarded and the tubes drained on glass fiber filter disks for five minutes. The glycogen was resuspended in 1 ml ETOH and recentrifuged for 10 minutes, the supernatant discarded and the tubes again drained on glass fiber paper. The glycogen was then dissolved in 2 ml of distilled water and centrifuged five minutes. The supernatant was decanted, and aliquots of this supernatant diluted according to the amount of tissue originally present in the sample. Duplicate 1-ml

aliquots of this second dilution were used for the chemical glycogen determination.

b. TCA-soluble glycogen

In order to isolate the TCA-soluble glycogen, the tubes stored in the deep freeze were brought to room temperature, 4 ml of 95% ETOH added and the tubes allowed to stand at room temperature for one hour and then placed in the refrigerator at 4°C overnight. The next day the precipitated glycogen was recovered and washed as described for the residual glycogen fraction.

2. Chemical Determination of Glycogen

The chemical determination of glycogen was carried out by a modification of the anthrone method of Roe[105].

a. Reagents

(1) Anthrone reagent: 100 mg anthrone (Nutritional Biochemicals Corporation) dissolved in 100 ml 79% H_2SO_4 [83 ml concentrated H_2SO_4 made to 100 ml (when cold)].

(2) Glucose standards: Stock standard contained 120 mg glucose per 100 ml saturated benzoic acid (250 mg%). Two dilute working standards contained 24 and 48 µg glucose/ml, respectively.

b. Method

Duplicate 1 ml aliquots of the aqueous dilution of the tissue glycogens, of the glucose working standards,

and of water for blanks were pipetted into Coleman cuvettes (19 x 105 mm). The cuvettes were placed in an ice bath and 5 ml of the anthrone reagent added slowly. The solutions were mixed on the Vortex and immediately returned to the ice bath. The cuvettes were stoppered with vented rubber stoppers (a short length of capillary tubing inserted through the stopper), and placed in a boiling water bath for 15 minutes. The incoming flow of water was turned off to insure constant temperature of the bath.

The cuvettes were transferred to an ice bath for five minutes, wiped dry, and then placed in the dark for 15 minutes. The optical density was read at 620 mμ in a Coleman Jr. spectrophotometer. Due to variations in the anthrone reagent, the standard values obtained for each day were used to calculate the concentration of glucose present.

c. Calculations

The results in terms of glucose were converted to mg glycogen per gram of tissue, wet weight, by dividing by the factor 1.11.

3. Glycogen-C^{14} Assay

Radioactivity of the glycogen samples was determined by plating triplicate 0.2 ml aliquots of the initial 2 ml solution of glycogen and counting in a low background Tracerlab Omniguard as previously described (IV C).

V. In vivo Studies

A. Animal Preparation and Tissue Sampling

Female Sprague Dawley rats, weighing from 225 to 250 g were fasted overnight. Approximately one hour before starting the experiment the animals were given 5 ml of a 50% solution of powdered milk in 2% glucose. The animals were anesthetized with Nembutal (2.5 mg/100 g body weight); and 50 μc of uniformly labeled glucose-C^{14} (New England Nuclear, 50 μc in 2.8 mg glucose) in 0.75 ml aqueous solution was injected either via the tail or the femoral vein. Using the time of injection of the C^{14} glucose as zero time, samples of red and white muscle were dissected from the muscles indicated within 15-30 minutes. Samples of red and white muscle were obtained from the following muscles:

Red muscle	White muscle
caudofemoralis	semimembranosus
semitendinosus	semitendinosus
semimembranosus	gastrocnemius
soleus	

The semimembranosus, semitendinosus and gastrocnemius muscles in the rat are mixed muscles, and on exsanguination, areas of red and white muscle are distinguishable. In these in vivo experiments in which the muscle was taken in biopsy, differentiation of muscle color was obscured by blood in the surgical area as well as in the vessels of the muscle and it was imperative that verification of the predominating fiber type be made by histochemical techniques. The soleus and caudofemoralis, macroscopically, are red muscles.

The strips of dissected muscle, 3-4 mm x 40 mm, were suspended between two clamps and quickly plunged into liquid nitrogen at -140°C or a freezing mixture of isopentane cooled to liquid nitrogen temperature. The clamps were removed in 20-40 seconds or when the bubbling of the freezing mixture had ceased, the muscle was detached and then quickly transferred to small tubes chilled to dry ice temperature. Samples of gastrocnemius and soleus were often too short to fit in the clamps. These were placed on small pieces of aluminum foil and quickly thrust into the freezing solution.

B. Tissue Preparation

Since a representative portion of the frozen muscle was needed for glycogen and histochemical determinations, the muscle was cut into sections in a cryostat. The temperature of the working area was maintained at -18 to -20°C which permitted manipulation of the tissue without contamination by moisture condensing on the frozen muscle. About 2/3 of the sample was cut into small pieces and saved for the determination of TCA-soluble and residual glycogen and the assay of C^{14} activity in these two fractions as previously described (IV D 2 and 3). About 1/3 of the sample was allocated for the histochemical determination of succinic dehydrogenase. In later experiments portions of the muscle samples were saved for the quantitative measurement of succinic dehydrogenase activity. The tubes containing the tissue aliquots were corked, placed in plastic freezer bags to prevent dehydration, and stored at dry ice temperature.

VI. Histochemical Techniques

A. Preparation of Tissue for Sectioning

Samples obtained from the *in vivo* glycogen-C^{14} experiments were removed from the dry ice and brought to -18 to -20°C in the cryostat.

The small pieces of tissue were mounted on the tissue holders by inserting from 2-4 mm of the frozen tissue into a punched depression in a small cork disk. The disk had previously been placed on a small piece of sponge saturated with water and frozen on the metal tissue holder. The tissue was immobilized in the cork by carefully placing small drops of water at the base of the tissue and in the depression and allowing it to freeze. Thawing of the tissue was prevented by holding a small piece of dry ice against the tissue.

Sections of the muscle were mounted on cover slips and incubated in appropriate media.

B. Succinic Dehydrogenase

Determination of succinic dehydrogenase was carried out according to the method of Nachlas *et al*[81] using nitro-blue tetrazolium, (2,2'-di-p-nitro-phenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4'-biphenylene] ditetrazolium chloride) (Nitro-BT). This method is based on the principle that tetrazolium salts in aqueous solution can serve as electron acceptors in the various metabolic dehydrogenase reactions and be converted to the insoluble formazan, a highly colored product, which is deposited at the site of enzyme activity in the tissue. There are various theoretical considerations for the choice of nitro-blue tetrazolium since its formazan has desirable characteristics of

low lipid solubility, high substantivity or affinity for protein, stability to light, and high color intensity.

1. Reagents

   a. Stock buffered succinate: Equal volumes of 0.2 M phosphate buffer, pH 7.4-7.6, and 0.2 M sodium succinate were mixed.

   b. Aqueous solution of Nitro-BT (1 mg/ml).

   c. Glycerine jelly mounting medium: 15 g white gelatine was dissolved in 100 ml distilled water with moderate heating. 100 g glycerol was added and the solution warmed for 5 minutes. Two drops of phenol were added as a preservative.

   d. Polyvinyl pyrollidone.

2. Substrate incubation medium

This reagent was prepared by combining 10 ml of the stock buffered succinate and 10 ml of the Nitro-BT (1 mg/ml). A control medium was made by combining 5 ml of phosphate buffer, pH 7.4-7.6, 5 ml of distilled water and 10 ml of Nitro-BT. One ml aliquots of substrate and control media were frozen and kept at -20°C at the time of analysis. 75 mg of polyvinyl pyrollidone (PVP) was added to the substrate and control media. The PVP served to increase the osmotic pressure of the media and is thought to protect the structure of the tissue.

3. Method

Tissue sections 6-8 mμ thick, mounted on cover slips,

were dried in the air for 2-3 minutes at room temperature.

The sections were placed on a rack of applicator sticks mounted on the bottom of Petri dishes. A small piece of sponge, moistened with water, was also placed in the dishes to maintain humidity. The tissues were then covered with the incubation or control medium, the Petri dishes closed and the sections incubated 15-30 minutes at 37°C.

The sections were then rinsed in saline, fixed in a solution of 10% formal saline (10 ml 40% formaldehyde in 0.9% saline) for 10 minutes, rinsed in saline and mounted in glycerine jelly.

C. Phosphorylase

The histochemical determination of phosphorylase was adapted from the method of Takeuchi and Kuriaki(119).

1. Reagents

a. Incubation medium:

Glucose-1-PO_4, 45 mg;

Adenosine monophosphate, 15 mg;

Sodium fluoride, 30 mg;

Glycogen, 3.0 mg;

Water, 15 ml.

A control medium containing the above reagents with the exception of substrate (glucose-1-PO_4) was also prepared.

1.5 ml aliquots of the substrate and control solutions were pipetted into small tubes and stored at -20°C. One ml 0.1 M acetate buffer (pH 5.6-6.0) and 0.5 ml of absolute

ethanol were added to 1.5 ml of substrate and control media immediately before use.

b. Gram's iodine: A stock solution of 1 part iodine, 2 parts KI and 30 parts distilled water was diluted 1:10 with water.

2. Method

Frozen sections were cut at 8-16 μ in the cryostat and mounted on cover slips, immediately covered with media (not allowed to dry) and incubated 15-30 minutes at 37°C. The tissues were transferred through 40% ETOH, 20% ETOH, water and dried in the oven at 37°C. After fixation in absolute ethanol for 3 minutes and drying in air at room temperature, the tissues were immersed in dilute Gram's iodine for 2-3 minutes. The cover slips were mounted in glycerine jelly in a solution of Gram's iodine:glycerine jelly (1:5). Newly-formed polysaccharide stained a deep blue. However, this color faded in two or three days, and if a more permanent preparation was desired, the sections were carried through the periodic acid-Schiff reaction.

D. UDPG-Glycogen Transglucosylase

UDPG-glycogen transglucosylase activities were demonstrated by the method of Takeuchi and Glenner[118].

1. Reagents

Incubation medium:

Glycogen, 10 mg;

EDTA, 20 mg;

Glucose-6-PO_4, 10 mg;

Water, 14 ml.

1.4 ml aliquots of this medium were stored at -20°C. At the time of analysis, two of the frozen aliquots were thawed and 1 ml tris buffer (0.2 M, pH 7.4) and 0.1 ml absolute ethanol added. Three mg UDPG was added to one aliquot as substrate, the other aliquot serving as a blank.

2. Method

Frozen sections cut 16-20 μ thick in the cryostat were incubated one-half to one hour in the substrate and control media at 37°C. The excess incubation medium was drained off. The sections were placed in diluted Gram's iodine solution until a red-brown coloration appeared and then mounted in iodine-glycerine jelly.

VII. Succinic Dehydrogenase-Formazan Extraction Method

The amount of succinic dehydrogenase activity in muscle samples was determined by incubating an aliquot of muscle homogenate in a buffered substrate solution containing a tetrazolium salt and quantitating spectrophotometrically.

A. Reagents

0.1% INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride], aqueous solution.

0.2 M phosphate buffer, pH 7.4-7.6.

0.1 M sodium succinate (substrate).

B. Method

A 1% weight/volume homogenate was made in phosphate buffer,

pH 7.4-7.6 (all-glass homogenizer).

Incubation media for the sample and its control were prepared by combining:

1 ml phosphate buffer, pH 7.4-7.6, and

1 ml 0.1% INT.

One ml of sodium succinate was added to the incubation mixture and 1 ml of water to the control. The mixture was incubated 15 minutes at 37°C in air. 0.1 ml of 30% TCA was added to terminate the reaction.

Seven ml ethyl acetate was pipetted into the samples, the tubes were shaken vigorously and then centrifuged five minutes at low speed. Approximately six ml of the supernatant was removed and the optical density read in a Coleman Jr. spectrophotometer at 490 mμ. The amount of color in the control, due to non-specific reduction of the tetrazolium salt, was subtracted from the sample value.

A standard curve was prepared using a commercial preparation of INT formazan (Nutritional Biochemicals Corporation). The curve was linear from 5-50 μg of INT Formazan/7 ml of ethyl acetate. INT was selected as the tetrazolium salt because unlike Nitro-BT its formazan has a low affinity for tissue protein (substantivity) and is readily extracted into ethylacetate.

VIII. Protein Determination. Adapted from the biuret method of Weichselbaum[124].

A. Reagents

1. Biuret copper reagent: 9 g Na-K tartrate (Rochelle salt), 3 g $CuSO_4.5H_2O$ and 5 g KI. The Rochelle salt was dissolved in about 400 ml of 0.2 N NaOH in a one-liter

volumetric flask. The $CuSO_4$ was added and stirred until completely dissolved. The KI was then added and the solution made to volume with 0.2 N NaOH.

2. Biuret reagent blank: This solution contained all the reagents listed above with the exception of $CuSO_4$.

B. Method

One-tenth ml of the supernatant from homogenized tissues, 0.9 ml of 0.05 N NaOH, and 2.0 ml of biuret reagent were combined for the biuret reaction. A blank was prepared using the biuret reagent blank instead of the copper reagent. The tubes were mixed thoroughly, incubated 30 minutes at 37°C and then read in the Beckman DU at 545 mμ. The optical density of the blank was substracted from the sample value.

A standard curve was set up using dilutions of Lab-trol serum (Dade) and was found to be linear from 0.66 to 2.64 mg of protein.

IX. Statistical Analysis

Statistical analysis of the results was carried out with either Student's t test for comparison of means or on the basis of paired observations. Differences were not considered significant unless the P value was at the 5% level or less.

RESULTS

I. Histochemical Characterization of Muscle Samples

A. Succinic Dehydrogenase

1. Frozen Sections

As previously indicated, the muscles used in these investigations were made up of varying proportions of red and white fibers. To characterize the type of fiber predominating in the muscles used, sections of frozen tissues were stained histochemically for succinic dehydrogenase. In plates I, II, and III, representative sections of the various muscles are shown. In those muscles designated as white, the majority of fibers appear larger and lighter staining with relatively few (usually 25% or less) staining more intensely and smaller in diameter. In the caudofemoralis and sections from red muscle of the semimembranosus and semitendinosus, generally three types of fiber are found, large and pale, small and dark, and fibers of intermediate color density and size. However, in comparison to white muscle, the difference in the number of dark and light fibers is striking. The soleus consists of dark staining fibers of rather uniform diameter with some of the fibers being more deeply staining than others.

The degree of staining is correlated with mitochondrial density, succinic dehydrogenase being primarily an enzyme associated with these subcellular components. The location of Nitro-BT formazan in the interfibrillar area corresponds

to the known location of mitochondria in muscle. In the soleus and in some red fibers of other muscles, large dark areas appear adjacent to the sarcolemma. This location of succinic dehydrogenase activity has been shown to be due to the presence of larger, or giant, mitochondria in distinction to the smaller mitochondria in the interior of the fiber[90].

Certain difficulties are inherent in characterizing the relative numbers of red and white fibers and attempting to correlate these results with metabolic activity. First of all, counting the numbers of individual fibers is a tedious procedure. However, this has been done by George and Talesara[33] who found a direct correlation between the relative number of narrow (red) fibers and the amount of formazan produced. Another factor is the presence of fibers of an intermediate size and degree of staining. In addition, muscle is a particularly difficult tissue to obtain, freeze, store, and section under optimal conditions necessary to produce fresh frozen sections which are consistently of excellent quality. In initial experiments, sections cut from representative pieces of muscles used in the experiments were stained for succinic dehydrogenase with Nitro-BT and graded on an arbitrary scale according to the numbers of small, dark staining (red) fibers present: 1+ consisted of approximately 25% or less red fibers; 2+, 25-50%; 3+, 50-75%; and 4+, over 75% red fibers. White muscle from the gastrocnemius, semitendinosus and semimembranosus consistently fell

within the 1+ to 2+ range of histochemical evaluation. Sections of red muscle from the semitendinosus, semimembranosus, caudofemoralis and soleus were classified in the 3 and 4+ range. However, because of the previously mentioned difficulties, it seemed of value to adopt an easier and perhaps more reliable method of evaluating the degree of "redness" of muscle.

2. Formazan Extraction

Although a manometric technique was one possible approach, a method based on tetrazolium salt reduction in homogenates seemed to offer more sensitivity since only 10 to 15 mg of tissue is required. In the method developed an aliquot of muscle homogenate was incubated in a buffered medium containing succinate as substrate, and the INT formazan extracted in ethyl acetate and quantitated colorimetrically. The results of the extraction procedure were compared to the histochemical evaluations determined on the same tissue samples. The comparisons are given in Table III. It appears that the histochemical evaluation of 1+ and 2+ cannot be distinguished by the formazan extraction method, since the values for 1+ sections (3.9 to 8.9) were included in 2+ sections (2.3 to 10.6). However, a marked difference was found in 3+ and 4+ tissues; the formazan production averaged about 2.5 to 3 times that of 1+ and 2+ tissues.

TABLE III

Correlation of the Relative Numbers of Red Fibers as Determined on Frozen Sections Stained for Succinic Dehydrogenase (Nitro-BT) and the Amount of INT Formazan Produced by Homogenates of the Same Muscle Sample

N[1]	H-C Evaluation	μg Formazan/mg Tissue
10	+	6.1 ± 0.57[2] (3.9 - 8.9)[3]
9	++	7.8 ± 1.11 (2.3 - 10.6)
13	+++	18.8 ± 1.3 (12.4 - 23.4)
4	++++	20.0 ± 1.2 (16.9 - 22.9)

[1]Number of observations

[2]Standard error of the mean

[3]Range

B. Phosphorylase

The reaction for phosphorylase is visualized by staining the newly formed polysaccharide with iodine. Under the conditions of incubation with glucose-1-PO_4 and in the presence of 20% alcohol (presumably precipitates soluble polysaccharide formed at the site of enzyme reaction), sufficient polysaccharide is formed within five minutes incubation to produce an intense blue stain. The product formed is assumed to be amylose or straight chain polysaccharide since highly branched native glycogen stains a brown or reddish brown with iodine. Fibers staining a purplish brown are also seen. These differences in coloration are probably due to the presence of newly synthesized glucosyl chains of varying length on terminal non-reducing branches of glycogen.

Serial sections stained for phosphorylase activity, in general, display the reciprocal relationship with succinic dehydrogenase observed by Dubowitz and Pearse[24]. That is, the larger fibers give a more intense phosphorylase reaction and contain less succinic dehydrogenase than do the small. Fibers of an intermediary degree of stain for succinic dehydrogenase were also intermediate between large and small fibers in phosphorylase activity. Fibers of the soleus were quite uniform in appearance. Control sections, incubated without glucose-1-PO_4, were stained pale yellow with iodine and showed no traces of blue coloration. Following malt diastase digestion (1% in phosphate buffer, pH 6.8, 15 minutes at 37°C) which removed the formed polysaccharide, the sections were negative to iodine stain. (Plate IV)

C. Transglucosylase

Histochemical preparations for transglucosylase activity were generally not too satisfactory. In contrast to the blue color of the phosphorylase reaction, the final product of transglcosylase activity in tissue sections stains a reddish brown with iodine. Three serial sections were made and stained for succinic dehydrogenase, phosphorylase and transglcusylase. Often the section stained for transglucosylase had to be compared closely with its serial phosphorylase and succinic dehydrogenase sections to demonstrate differential fiber staining. However, the smaller fibers (high succinic dehydrogenase and low phosphorylase) generally stained darker than the large fibers, indicating a higher transglucosylase activity. Addition of insulin, uridine triphosphate, and increased amounts of glucose-6-PO_4 failed to improve the quality of the transglucosylase reaction. (Plates V,VI)

II. Quantitation of Phosphorylase and Transglucosylase Activities in Muscle Homogenates

Histochemical reactions in rat muscle have suggested that phosphorylase and transglucosylase enzymes are located in different fibers. However, it has not been determined whether this difference exists in rat muscle when the enzyme activities are assayed by standard biochemical techniques. The assay procedures selected in the work presented here have been commonly used for the demonstration of phosphorylase and transglucosylase activities in homogenates of various tissues.

A. Phosphorylase

Samples of semimembranosus and caudofemoralis muscle were used in these determinations. Aliquots of the supernatant from the 800 x g fraction of homogenates were assayed for total phosphorylase (AMP present) and for active phosphorylase (AMP absent). Table IV shows that the enzyme activity was about 3-fold higher in white than in red muscle, both in total and active phosphorylase. Seventy-eight

TABLE IV

Total Phosphorylase (+AMP) and Active Phosphorylase (-AMP) Activities of Supernatant Fractions of Homogenates of Predominantly Red and White Muscle Fiber Groups from the Semimembranosus Muscle (μg phosphorus/g tissue, wet weight/min)

	N[1]	Total Phosphorylase (+AMP)	Active Phosphorylase (-AMP)
White muscle	16	2845 ± 300[2]	2189 ± 314
Red muscle	17	902 ± 247	673 ± 212
P		< .001	< .001

[1]Number of experiments; [2]Standard error of the mean

per cent of the total enzyme activity was in the active form in white muscle as compared to 75% in the red. The results are expressed as μg of phosphorus produced per gram of muscle wet weight per minute since no difference was found in the two types of muscle either in dry weight or protein content of the supernatant as determined by the biuret method (Table V).

TABLE V

Protein Concentration (Biuret Method) of Supernatant Fraction of Homogenates and Per Cent Dry Weight of Red and White Muscle

	N[1]	Red Muscle	White Muscle	P[2]
Protein mg/g tissue, wet weight	14	57.6 ± 0.98[3]	56.5 ± 0.94	> .10
% dry weight	9	23.7 ± 0.37	23.7 ± 0.54	> .10

[1]Number of experiments
[2]P, red vs white muscle
[3]Standard error of the mean

Although phosphorylase is a soluble enzyme of the sarcoplasm, there is some evidence that the enzyme may be bound to particulate glycogen(112). Table VI shows that if whole muscle homogenate is used in the assay rather than the supernatant fraction, white muscle has a 2-fold higher content of total phosphorylase and about a 3-fold higher content of active phosphorylase than does red. The active phosphorylase in white muscle comprises about 68% of the total enzyme activity while in red only 50%. The lower values found in all phosphorylase

fractions of whole homogenate would seem to reflect the dilution effect due to the lower concentration of enzyme in the insoluble components of the tissue.

TABLE VI

Total Phosphorylase (+AMP) and Active Phosphorylase (-AMP) Activities of Whole Homogenates of Predominantly Red and White Muscle Fiber Groups from the Semimembranosus Muscle (μg phosphorus/g tissue wet weight/minute)

	N[1]	Total Phosphorylase (+AMP)	Active Phosphorylase (-AMP)
White muscle	9	1412 ± 285[2]	944 ± 265
Red muscle	9	642 ± 95	331 ± 54
Average difference (W - R)		+770 ± 248[3]	+614 ± 242
P[4] for difference		< .02	< .05

[1]Number of experiments

[2]Standard error of the mean

[3]Standard error of the difference

[4]Statistical analysis on the basis of paired observations

B. Transglucosylase Activity in Muscle Homogenates

Transglucosylase activity, as determined by the method of Leloir and Goldemberg[68], involves two enzymatic steps, and although this procedure increased possible sources of error, the degree of consistency was very high. Six assays were run in duplicate and a comparison made between the first and second samples. Analysis of the results shows that there is no significant difference between the two samples (P for the difference, > .10).

Muscles used in transglucosylase assays, in addition to the semimembranosus, were the semitendinosus, gastrocnemius and soleus. Table VII shows the levels of activity found in paired red and white muscle samples.

TABLE VII

Uridine Diphosphoglucose Glycogen Transglucosylase Activity of Predominantly Red and White Muscle Fiber Groups (μmoles UDP/g tissue wet weight/minute)

N[1]	Red muscle	White muscle	Average difference (R - W)	P[2] for difference
	Semimembranosus			
5	4.90 ± 0.25[3]	3.47 ± 0.16	+1.46 ± 0.14[4]	< .001
	Semitendinosus, gastrocnemius and soleus			
8	3.94 ± 0.26	2.56 ± 0.11	+1.41 ± 0.20	< .001

[1]Number of experiments
[2]Statistical analysis on the basis of paired observations
[3]Standard error of the mean
[4]Standard error of the difference

Transglucosylase activity is about 30% higher in red fibers than in white fibers of the semimembranosus muscle. The results from the semimembranosus were tabulated separately because this muscle was the principal source of tissue in phosphorylase activity determinations. However, the higher activity in red muscle was also found when white muscle from gastrocnemius, red muscle from the soleus, and red and white fiber groups from the semitendinosus were analyzed. When mean

values for all red versus all white muscle were compared the difference is significant at the 1% level (red, 4.31 $\pm$ 0.22 s.e. μmoles UDP/g/min; white, 2.86 $\pm$ 0.14, n=14). Values for red muscle are about 35% higher than for white. The results from homogenates of muscle, then, agree with the histochemical observations in showing that white muscle fibers have higher phosphorylase while red muscle fibers have higher transglucosylase activity. Since glycogen is the substrate of phosphorylase and the product of transglucosylase, and in view of the reported higher content of glycogen in white muscle, it seemed that investigation of the incorporation of C^{14}-glucose into glycogen might explain some of the differences in the metabolic patterns of white and red muscle.

III. In vitro Experiments--Comparison of Red and White Muscle

Preliminary in vitro results from our laboratory using acetoacetic acid-3-C^{14} as a substrate in the medium for muscle incubation showed the specific activity to be 1.5 times higher in the glycogen fraction of the white than in that of the red muscle[1]. In these same experiments the total glycogen content determined immediately after decapitation of the animal was higher in white muscle. However, after 2 hours incubation in the presence of acetoacetic acid, the levels of glycogen were significantly lower in white muscle than in red(5). These results would seem to indicate that glycolysis is more active in white than in red muscle.

A. Specific Activities of Glycogen Fractions

In the experiments reported here the two types of muscle

[1]Unpublished results.

were incubated in Krebs bicarbonate buffered medium plus 150 mg % glucose and containing 1 μc glucose-C^{14} (UL) per ml of medium. It was found that the specific activities of the glycogen fractions were higher in red muscle in all time periods studied. Results of individual experiments are illustrated in figures 5-8. The specific activities of residual and TCA-soluble glycogen are plotted against the time of incubation; red muscle being indicated by clear bars and white muscle by the stippled area. For the time periods studied glycogen samples from red muscle averaged 2 to 16 times the specific activities of samples from white muscle. Average values for specific activities for the six experimental periods are given in Table VIII. In addition, Table VIII also indicates that the specific activities were significantly higher in the TCA-soluble glycogen than in the residual glycogen of red muscle in four of the seven sample times (calculated on the basis of paired observations). Ten minutes after the addition of substrate to the muscle, the specific activities of the two fractions were similar. The 30-minute sample consisted of only four experiments. Differences for the 60-minute period approached the 5% level of significance. No such differences were found in white muscle as the specific activities of both glycogen samples were similar at all time periods studied.

When ratios of specific activities of red and white muscle were compared (Table IX) it was noted that the ratios decreased with time of incubation. Initially, the specific activities for the TCA-soluble and residual glycogen are above 14.5 times higher in red muscle than in white. After two hours incubation the ratio decreased to about 2.5. In Figure 9 the specific activities for the two glycogen fractions

Figure 5. Comparison of the specific activities of residual glycogen isolated from red muscle (clear bars) and white muscle (stippled bars) after incubation of the tissue in the presence of glucose-C^{14} (UL) for the various time periods indicated, Krebs bicarbonate buffer, pH 7.4, plus 150 mg % glucose. Results obtained from individual experiments are indicated. Experiments 1, 2 and 3.

FIGURE 5

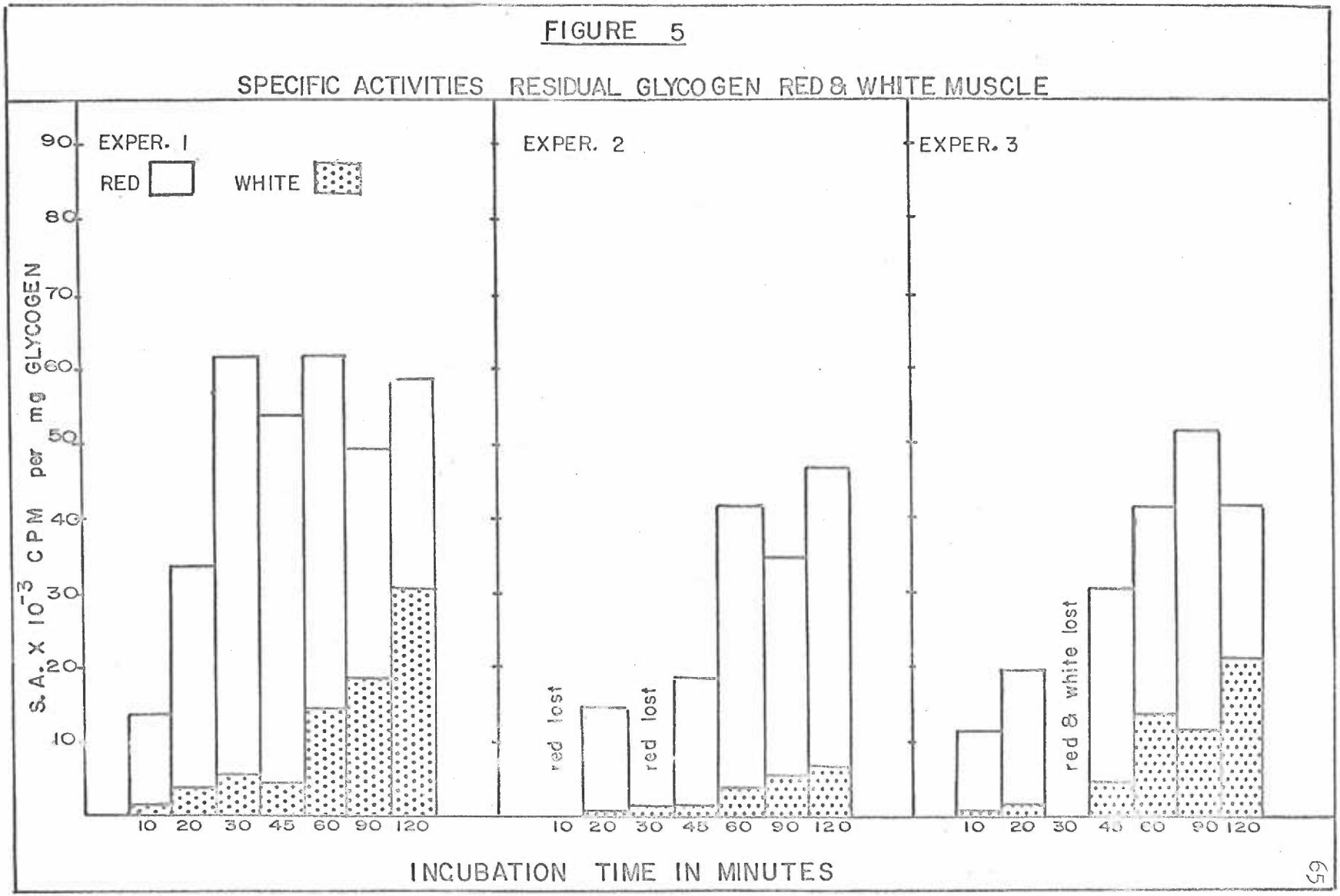

SPECIFIC ACTIVITIES RESIDUAL GLYCOGEN RED & WHITE MUSCLE
EXPER. 1
RED
WHITE
EXPER. 2
EXPER. 3
S.A. X 10^{-3} CPM per mg GLYCOGEN
90
80
70
60
50
40
30
20
10
red lost
red lost
red & white lost
10 20 30 45 60 90 120
10 20 30 45 60 90 120
10 20 30 45 60 90 120
INCUBATION TIME IN MINUTES

Figure 6. Comparison of the specific activities of residual glycogen isolated from red muscle (clear bars) and white muscle (stippled bars) after incubation of the tissue in the presence of glucose-C^{14} (UL) for the various time periods indicated, Krebs bicarbonate buffer, pH 7.4, plus 150 mg % glucose. Results obtained from individual experiments are indicated. Experiments 4, 5 and 6.

FIGURE 6

SPECIFIC ACTIVITIES RESIDUAL GLYCOGEN RED & WHITE MUSCLE

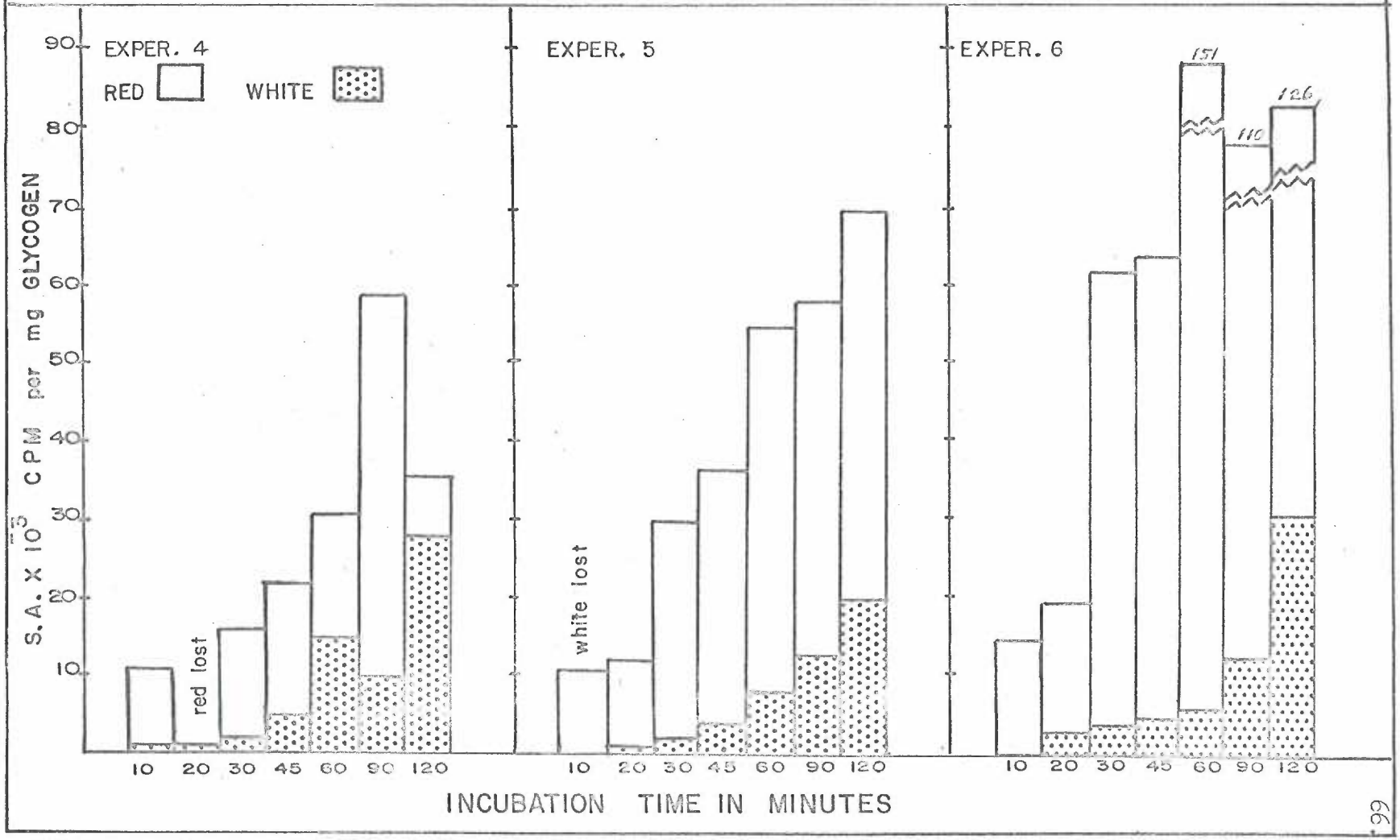


Figure 7. Comparison of the specific activities of trichloroacetic acid-soluble glycogen isolated from red muscle (clear bars) and white muscle (stippled bars) after incubation of the tissue in the presence of glucose-C^{14} (UL) for the various time periods indicated, Krebs bicarbonate buffer, pH 7.4, plus 150 mg % glucose. Results obtained from individual experiments are indicated. Experiments 1, 2 and 3.

FIGURE 7.

SPECIFIC ACTIVITIES TCA--SOLUBLE GLYCOGEN RED & WHITE MUSCLE

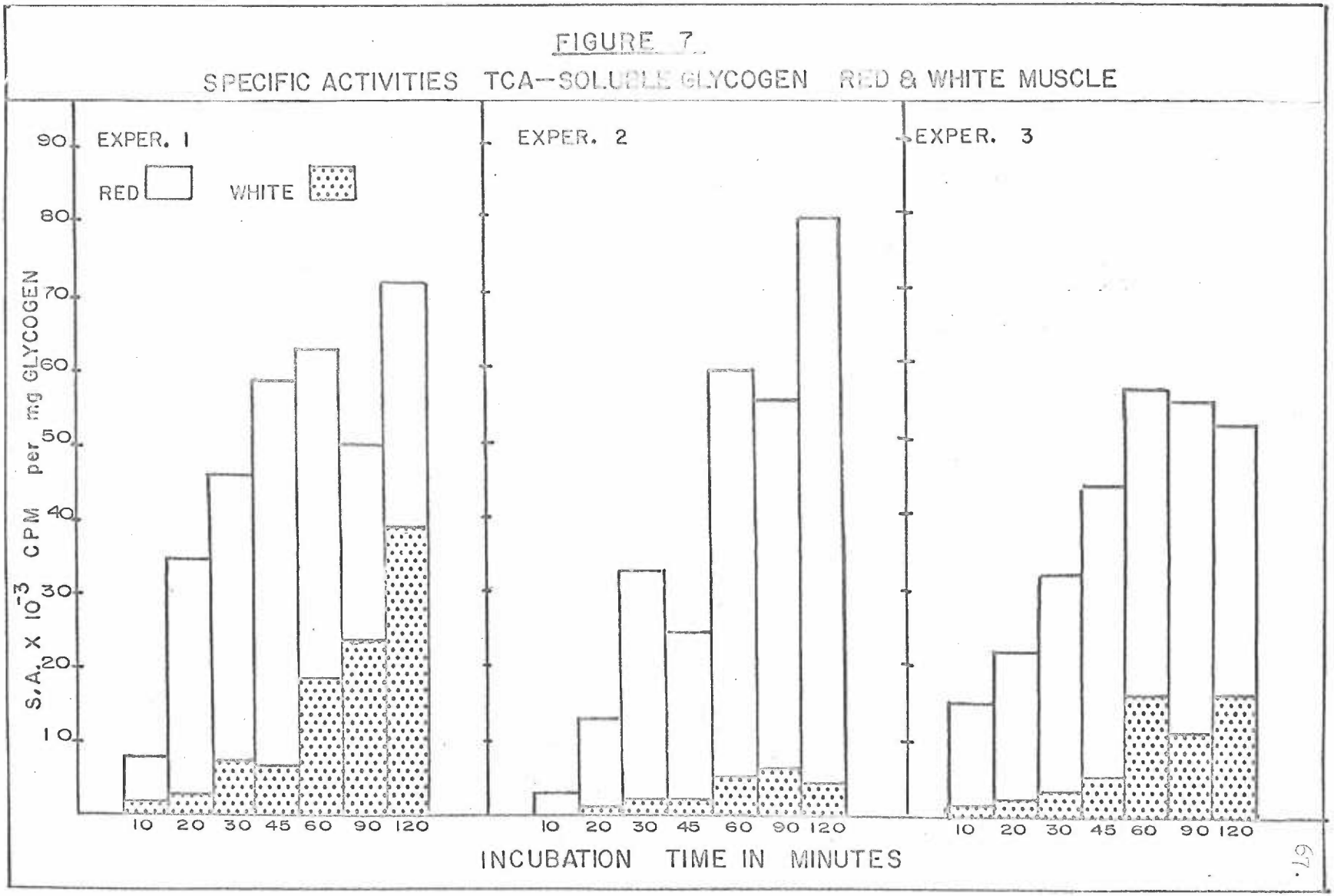


Figure 8. Comparison of the specific activities of trichloroacetic acid-soluble glycogen isolated from red muscle (clear bars) and white muscle (stippled bars) after incubation of the tissue in the presence of glucose-C^{14} (UL) for the various time periods indicated, Krebs bicarbonate buffer, pH 7.4, plus 150 mg % glucose. Results obtained from individual experiments are indicated. Experiments 4, 5 and 6.

FIGURE 8

SPECIFIC ACTIVITIES TCA-SOLUBLE GLYCOGEN RED & WHITE MUSCLE

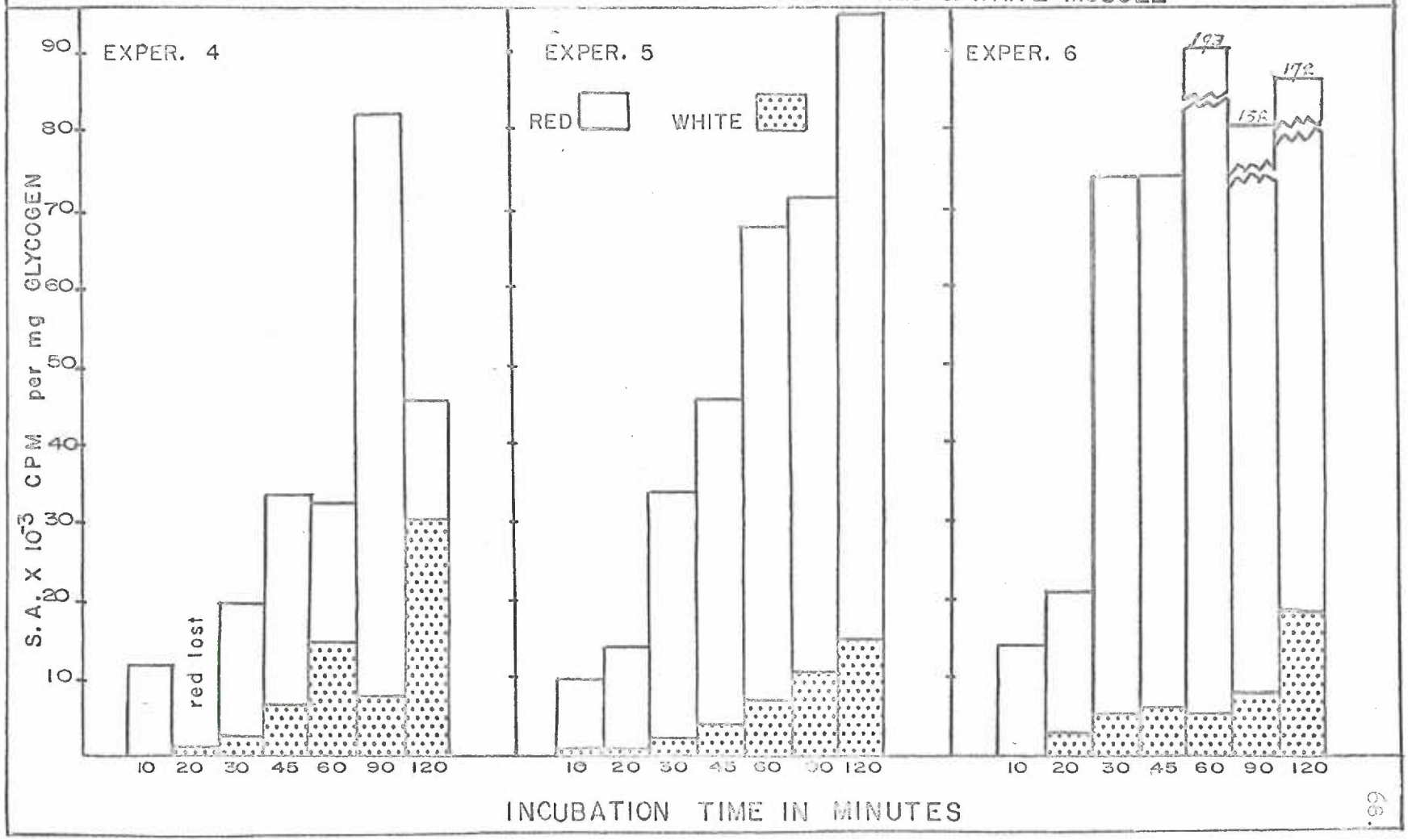


Figure 9. Comparison of the mean specific activities of residual and TCA-soluble glycogen isolated from red and white muscle after incubation in the presence of glucose-C^{14} (UL) for the various time periods indicated. The values indicated were taken from Table VIII.

FIGURE 9

COMPARISON OF MEAN SPECIFIC ACTIVITIES RESIDUAL AND TCA-SOLUBLE GLYCOGEN, RED AND WHITE MUSCLE

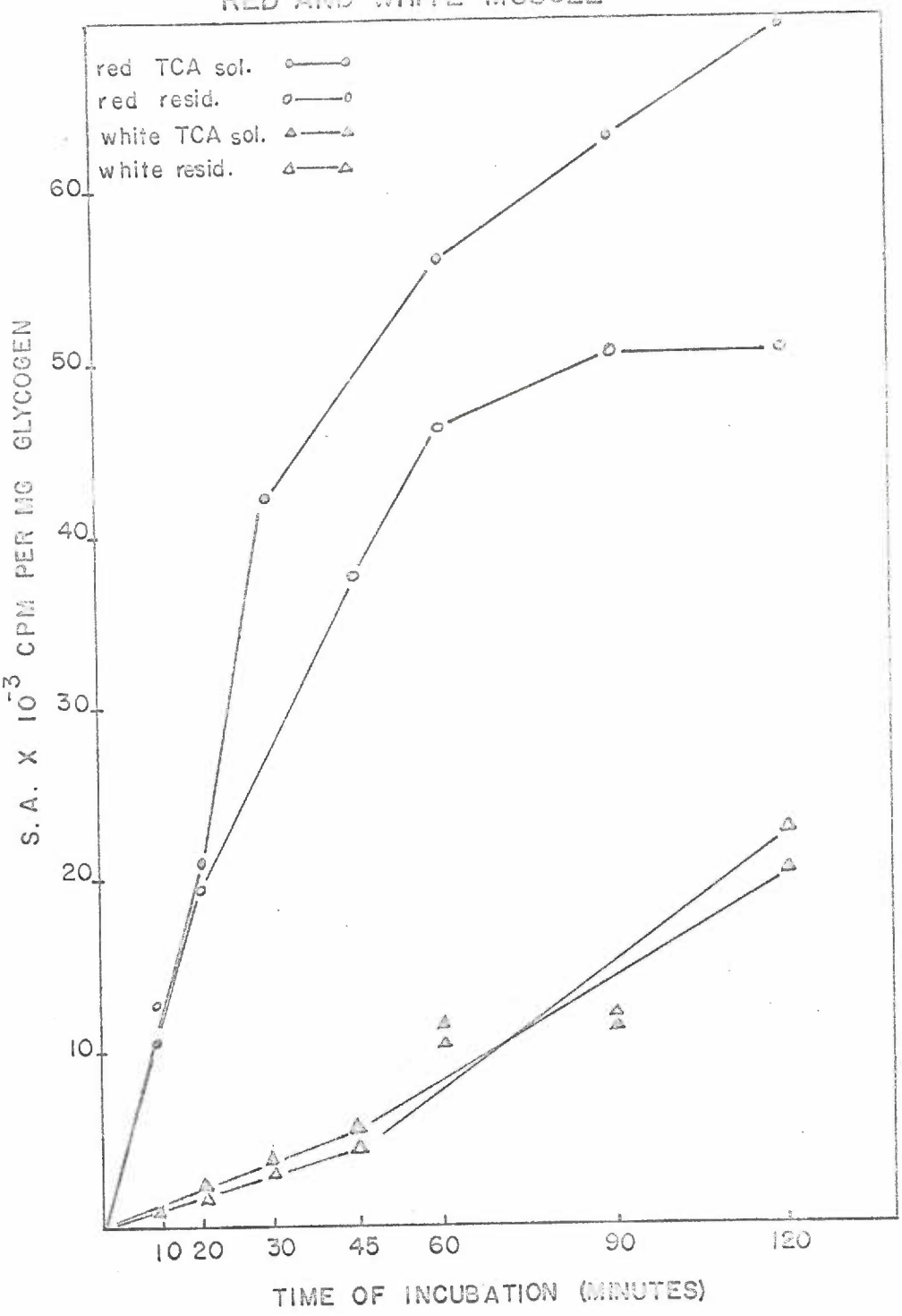


TABLE VIII

Comparison of Specific Activities (cpm/mg glycogen) of the Residual and TCA-soluble Glycogen in Red and White Muscle after Incubation in the Presence of 1 μc/ml Glucose-C-14 (UL) for Varying Time Periods, Krebs Bicarbonate Buffer, pH 7.4, plus 150 mg % Glucose

Minutes of Incubation	N[1]	Residual Glycogen	TCA Glycogen	Av. Difference (TCA-Res)	P[2] for Difference
		Red Muscle S.A. x 10^{-3}			
10	5	12.6 ± 2.5[3]	10.3 ± 1.8	-0.8 ± 1.5[4]	> .10
20	5	19.2 ± 4.1	21.0 ± 4.0	+1.8 ± 0.2	< .001
30	4	42.5 ± 11.5	42.0 ± 8.8	+0.5 ± 5.0	--
45	6	37.8 ± 7.3	47.0 ± 7.2	+9.2 ± 1.3	< .005
60	5	46.4 ± 5.4	56.0 ± 6.0	+9.6 ± 3.6	< .06
90	5	50.6 ± 4.3	63.0 ± 6.0	+14.0 ± 3.1	< .025
120	5	50.8 ± 6.1	69.4 ± 9.1	+18.6 ± 4.9	< .025
		White Muscle S.A. x 10^{-3}			
10	5	0.8 ± 0.4	0.8 ± 0.4	-0.2 ± 0.2	> .10
20	6	2.0 ± 0.5	1.8 ± 0.4	-0.2 ± 0.2	> .10
30	5	3.2 ± 0.8	4.0 ± 1.1	-0.4 ± 0.4	> .10
45	6	4.3 ± 0.5	5.2 ± 0.8	+0.5 ± 0.5	> .10
60	6	10.3 ± 2.0	11.1 ± 2.6	+0.8 ± 0.8	> .10
90	6	12.0 ± 1.7	11.3 ± 2.7	-0.7 ± 1.2	> .10
120	6	22.5 ± 3.7	20.5 ± 5.1	-2.3 ± 2.8	> .10

[1]Number of experiments

[2]Statistical analysis on the basis of paired observations

[3]Standard error of the mean

[4]Standard error of the difference

were plotted against time of incubation. Inspection of the slopes of the curves demonstrates that as the time of incubation increased, the rate of change in the amount of C^{14} label appearing in the glycogen fractions of white muscle increased while that of red muscle decreased. In red muscle the rate of change in the amounts of label incorporated into residual glycogen is lower than into TCA-soluble glycogen.

TABLE IX

Ratios of Specific Activities of Glycogen Fractions from Red and White Muscles Calculated at the Various Experimental Periods

Ratio: $\frac{\text{Glycogen S.A. in Red Muscle}}{\text{Glycogen S.A. in White Muscle}}$							
Minutes	10	20	30	45	60	90	120
N	5	5	4	6	5	5	5
TCA-soluble	13	12	10	9	5	6	3
Residual	16	9	14	9	4	4	2

Although the ratios of the specific activities of glycogen fractions of red and white muscle approached 2.5 after 2 hours incubation, the differences in specific activities between red and white muscle were significant for both glycogen fractions ($P < .05$ for residual and $P < .01$ for TCA-soluble glycogen [Table VIII]).

B. $C^{14}O_2$ Production

As an indication of metabolic activity of the tissues, the respiration of muscle samples was checked by determining the amount of

$C^{14}O_2$ produced. Table X shows the average values obtained in counts per minute per gram of tissue wet weight for the seven experimental periods.

TABLE X

$C^{14}O_2$ Production by Predominantly Red and White Muscle Fiber Groups, in vitro Experiments, Krebs Bicarbonate Buffer, pH 7.4, plus 150 mg % Glucose and 1 μc/ml Glucose-C^{14} (UL) (cpm/g tissue wet weight x 10^{-3})

Minutes of Incubation	N[1]	Red Muscle	White Muscle	Av. Difference (R - W)	P[2] for Difference
10	6	5.2 ± 1.2[3]	3.5 ± 0.7	-1.7 ± 0.7[4]	< .06
20	5	10.8 ± 2.3	8.2 ± 1.4	-2.2 ± 1.2	> .10
30	6	16.2 ± 2.9	10.5 ± 2.0	-5.7 ± 1.2	< .01
45	6	25.5 ± 4.3	19.6 ± 4.7	-8.0 ± 1.8	< .025
60	6	45.5 ± 9.9	27.0 ± 4.7	-18.5 ± 5.8	< .025
90	5	80.6 ± 17.7	36.7 ± 5.3	-42.8 ± 7.2	< .025
120	5	82.8 ± 9.8	58.3 ± 8.9	-44.3 ± 6.3	< .025

[1]Number of experiments

[2]Statistical analysis on the basis of paired observations

[3]Standard error of the mean

[4]Standard error of the difference

It can be seen that the over-all amounts of $C^{14}O_2$ produced from glucose-C^{14} labeled substrate was higher in red than in white muscle. This is in agreement with the concept that activity of the citric acid cycle is higher in red muscle. The results of the individual experiments are shown in figures 10 and 11. $C^{14}O_2$ production is plotted

Figure 10. Comparison of the $C^{14}O_2$ produced by red and white muscle during incubation in the presence of glucose-C^{14} (UL) for the various time periods. Experiments 1, 2 and 3.

FIGURE 10

CPM $C^{14}O_2$ PRODUCED BY RED AND WHITE MUSCLE

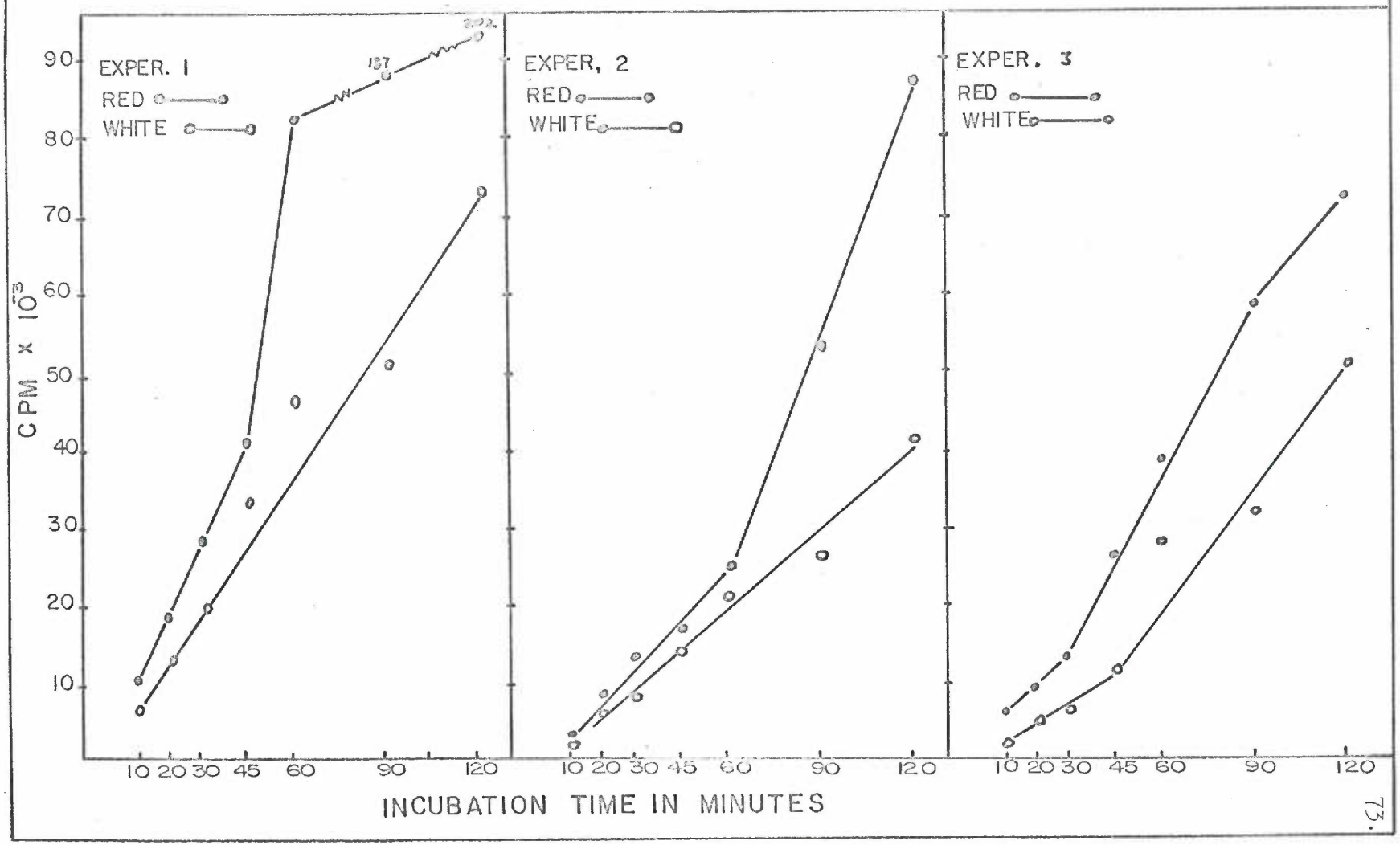
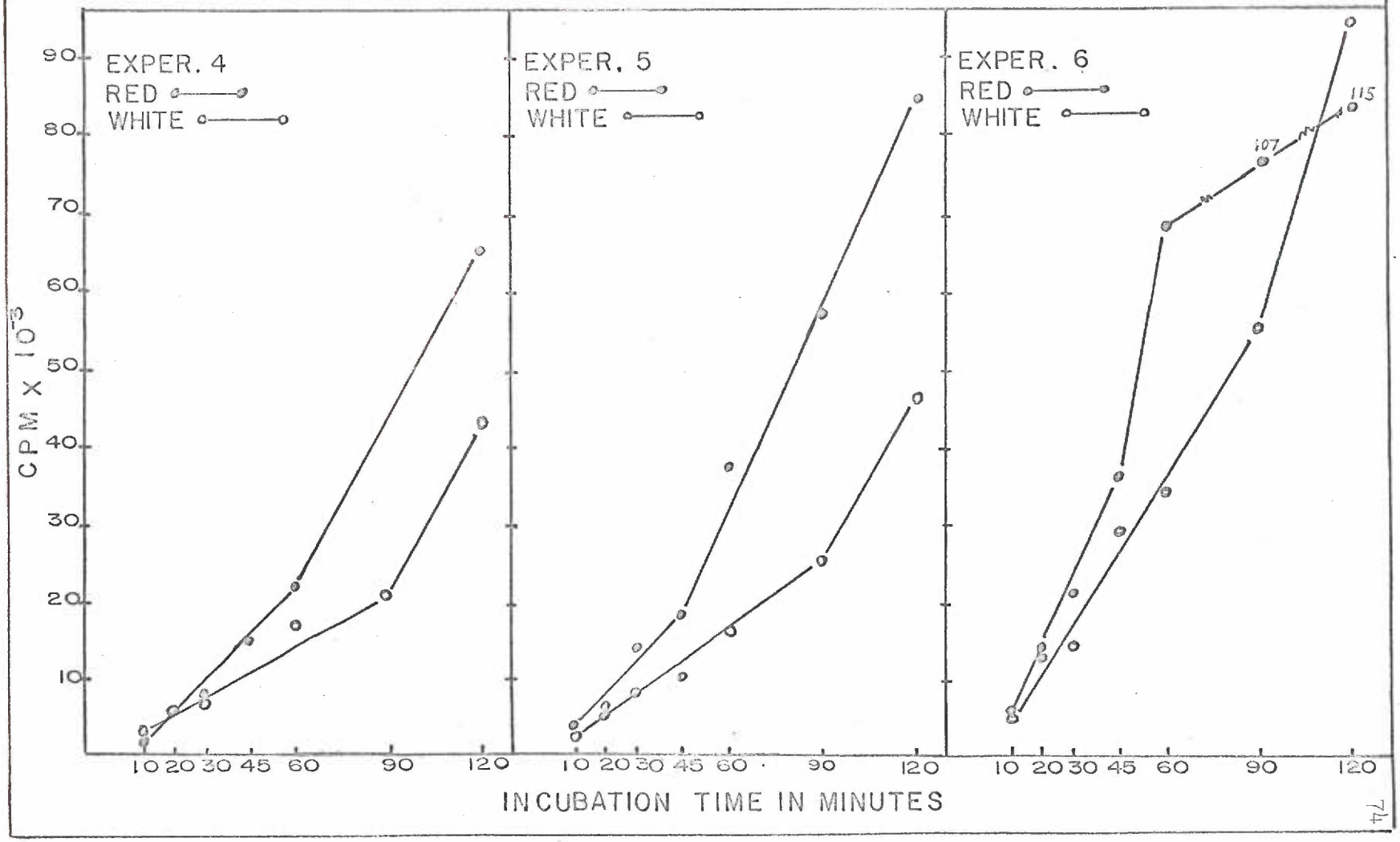


Figure 11. Comparison of the $C^{14}O_2$ produced by red and white muscle during incubation in the presence of glucose-C^{14} (UL) for the various time periods. Experiments 4, 5 and 6.

FIGURE II

CPM $C^{14}O_2$ PRODUCED BY RED AND WHITE MUSCLE



with respect to time of incubation for both types of muscle. The rates are generally linear over the early metabolic periods. However, the rates of production increased sharply in the red muscle after about 45 to 60 minutes incubation. In white muscle, if the rate increase appeared, it was generally later in time than for the red muscle, and the increase less striking. It is difficult to explain this change in CO_2 production. Possibly the manipulation of the muscle in preparing it for incubation may have injured some metabolic system which recovered after a period of incubation. At present there is no speculation as to the nature of the mechanism suggested by this change in rate of production of $C^{14}O_2$.

C. Levels of Total Glycogen

In agreement with previous observations, the level of total glycogen at equilibrium (control samples) is higher in white muscle (Table XI). During incubation, the levels of glycogen decreased in both types of muscle but the decrease was greater in white than in red muscle. After 90 minutes incubation the level of white muscle was significantly lower than in red. This difference was even more pronounced at the end of the two hour incubation. The decrease in glycogen levels during incubation was approximately 45% for white muscle as compared to only 25% for red muscle.

D. Levels of TCA-soluble and Residual Glycogen

Although the specific activities of the TCA-soluble and residual fractions of glycogen in red muscle were different, the actual levels of glycogen in these two fractions were similar. Table XII shows the amounts of the glycogen fractions in red and white

TABLE XI

Comparison of Levels of Total Glycogen (mg/g tissue wet weight) in Predominantly Red and White Muscle Fiber Groups

Minutes of Incubation	N[1]	White Muscle	Red Muscle	Av. Difference (W - R)	P[2] for Difference
Control	6	3.08 ± 0.32[3]	2.61 ± 0.23	+0.52 ± 0.14[4]	< .02
10	6	3.00 ± 0.30	2.82 ± 0.15	+0.16 ± 0.26	> .10
20	5	2.75 ± 0.31	2.90 ± 0.14	+0.02 ± 0.28	> .10
30	6	2.83 ± 0.15	2.50 ± 0.27	+0.22 ± 0.31	> .10
45	6	2.37 ± 0.20	2.70 ± 0.19	-0.58 ± 0.23	< .10
60	6	2.85 ± 0.29	2.77 ± 0.21	-0.64 ± 0.26	< .10
90	6	1.72 ± 0.04	2.26 ± 0.15	-0.46 ± 0.13	< .05
120	6	1.70 ± 0.36	1.95 ± 0.19	-0.50 ± 0.07	< .001

[1]Number of experiments

[2]Statistical analysis on the basis of paired observations

[3]Standard error of the mean

[4]Standard error of the difference

muscle at the end of the various incubation periods. The decrease in glycogen levels of red muscle over the two hour incubation period was 20% for the TCA-soluble and 40% for the residual glycogen fractions. The TCA-soluble glycogen of the white muscle, however, was significantly lower in the control, 20, 90 and 120 minute samples. The total decrease in glycogen of white muscle during incubation was 0.7 mg/g of tissue for both TCA-soluble and residual glycogen. In terms of per cent decrease from control levels the values are 54% for the TCA-soluble and 39% for the residual glycogen.

TABLE XII

Comparison of Levels of TCA-soluble and Residual Glycogen in Predominantly Red and White Muscle Fiber Groups after Incubation in Krebs Bicarbonate Buffer, pH 7.4, plus 150 mg % Glucose and 1 μc/ml Glucose-C-14 (UL)

Minutes of Incubation	N[1]	TCA-soluble Glycogen	Residual Glycogen	Av. Difference (Resid. - TCA)	P[2] for Diff.
		Red Muscle (mg glycogen/g tissue wet weight)			
Control	6	1.2 ± 0.14[3]	1.4 ± 0.20	+0.18 ± 0.38[4]	> .10
10	6	1.5 ± 0.12	1.4 ± 0.01	-0.12 ± 0.11	> .10
20	5	1.4 ± 0.11	1.5 ± 0.05	+0.18 ± 0.14	> .10
30	3	1.5 ± 0.05	1.4 ± 0.04	-0.10 ± 0.03	> .10
45	5	1.4 ± 0.10	1.4 ± 0.05	-0.02 ± 0.20	> .10
60	6	1.4 ± 0.15	1.5 ± 0.16	+0.03 ± 0.18	> .10
90	4	1.0 ± 0.16	1.2 ± 0.15	+0.22 ± 0.18	> .10
120	5	1.0 ± 0.07	1.0 ± 0.06	0	-
		White Muscle (mg glycogen/g tissue wet weight)			
Control	5	1.3 ± 0.22	1.8 ± 0.14	+0.54 ± 0.15	< .02
10	5	1.2 ± 0.13	1.8 ± 0.25	+0.56 ± 0.25	< .10
20	5	1.1 ± 0.17	1.7 ± 0.26	+0.52 ± 0.18	< .05
30	6	1.2 ± 0.08	1.6 ± 0.24	+0.37 ± 0.18	< .10
45	6	1.0 ± 0.06	1.4 ± 0.18	+0.42 ± 0.20	> .10
60	6	1.3 ± 0.14	1.5 ± 0.26	+0.18 ± 0.29	> .10
90	6	0.7 ± 0.07	1.1 ± 0.11	+0.45 ± 0.16	< .05
120	6	0.6 ± 0.10	1.1 ± 0.13	+0.43 ± 0.16	< .05

[1]Number of experiments

[2]Statistical analysis on the basis of paired observations

[3]Standard error of the mean

[4]Standard error of the difference

IV. In vivo Experiments

A. Specific Activities of Glycogen Fractions

In view of the demonstration of differences in glycogen metabolism between red and white muscle *in vitro*, a series of experiments was performed *in vivo* to determine the amount of C^{14} label from glucose-C^{14} appearing in the two fractions of glycogen. Animals were injected intravenously with approximately 50 μc glucose-C^{14} (UL), and various muscles or areas of muscle composed of predominantly red or white fibers were removed at biopsy and assayed for C^{14} activity in the TCA-soluble and residual glycogen fractions. The results from a series of 17 pairs of red and white muscle preparations from four animals are given in Table XIII.

TABLE XIII

Specific Activity of Glycogen Fractions of Red and White Muscle Removed from the Animal 15-30 Minutes after I.V. Injection of Approximately 50 μc Glucose-C-14 (UL)

		Specific Activity x 10^{-3}	
	N[1]	TCA Glycogen	Residual Glycogen
Red Muscle	17	1.47 ± 0.33[2]	1.40 ± 0.28
White Muscle	17	0.59 ± 0.08	0.49 ± 0.07
P		< 0.05	< 0.05

[1] Number of samples

[2] Standard error of the mean

The pairs of red and white muscle consisted of tissue from semimembranosus, semitendinosus, gastrocnemius, soleus and caudofemoralis

muscles. In agreement with the in vitro data, it was found that the specific activities of both glycogen fractions were higher in red than in white muscle, the TCA-soluble and residual glycogen activities being 2.5 and 2.8 times higher, respectively, in red muscle. In these in vivo experiments, there was no difference in the specific activities of TCA and residual glycogen fractions from either type of muscle.

B. Levels of TCA-Soluble, Residual and Total Glycogen

The levels of TCA-soluble and residual glycogen in these in vivo experiments are given in Table XIV. Levels of total glycogen are also given.

TABLE XIV

Levels of TCA-soluble, Residual and Total Glycogen in Red and White Muscle, in vivo Experiments (mg/g tissue wet weight)

	N[1]	Red Muscle	White Muscle	P[2]
TCA-soluble	17	1.23 ± 0.20[3]	1.52 ± 0.20	> .10
Residual	17	2.54 ± 0.01	2.61 ± 0.15	> .10
Average difference		$+1.05 \pm 0.22$[4]	$+1.09 \pm 0.15$	
P[5]		< .001	< .001	
Total glycogen	17	3.77 ± 0.21	4.13 ± 0.33	> .10

[1] Number of paired muscle samples

[2] P for the difference, red vs white

[3] Standard error of the mean

[4] Standard error of the difference

[5] P for the difference, TCA-soluble vs residual, paired observations

When differences between paired red and white muscle are analyzed, levels of residual glycogen are significantly higher than those

of TCA-soluble glycogen; the residual being higher by factors of about 2.0 in red and 1.7 in white muscle. However, the levels of the two glycogen fractions from red and white muscle are similar ($P > .10$ for both fractions). The levels of total glycogen are also similar for both types of muscle. These *in vivo* results differ from the *in vitro* data in that in the Warburg experiments there was no difference in levels of glycogen in the TCA-soluble and residual fraction in red muscle. The significance of these observations will be discussed in the following section.

DISCUSSION

I. Histochemical Evaluation of Red and White Muscle

Histochemistry is a relatively new tool available for studying biochemical reactions of tissues. Classical analytical techniques have yielded a wealth of biochemical information. However, the study of relationships of biochemical processes to cellular architecture and general function of a given cell or groups of cells can also be approached through histochemical techniques. Certain limitations are inherent in these techniques, especially for localization of enzyme activity. Many enzymes require co-factors, which may be at abnormally low concentrations in isolated tissues, and unless these co-factors are added in the incubation medium, little or no activity will be detected. Visualization of the end product of an enzyme reaction may present difficulties. In the phosphorylase and transglucosylase reactions the polysaccharide formed reacts directly with iodine and is easily located on microscopic examination of the tissue. With dehydrogenase enzymes such as succinic dehydrogenase visualization depends on the shunting of electrons from the normal flow along the electron transport chain to a tetrazolium salt, which, when reduced, forms an intensely colored and highly insoluble product. Theoretically a deficiency of any one enzyme or required co-factor in the chain preceding the level at which the tetrazolium salt accepts electrons can block this reaction, giving a negative or low result for the enzyme activity. This in effect is what Van Wijhe et al[122] found when they observed that histochemically α-glycerophosphate dehydrogenase (α-GPDH) was low in white muscle fibers. However, when homogenates of muscle

were assayed in the presence of an electron acceptor, menadione, a limiting DPNH diaphorase (dehydrogenase) was by-passed and white fibers reacted more strongly for α-GPDH activity than did red fibers. Whether or not this diaphorase is rate limiting *in vivo* is not known.

By histochemical techniques maximal activities of phosphorylase and transglucosylase were located in different fiber types, however, this dissociation does not seem logical. For, if glycogen synthesis is mediated by transglucosylase and glycogenolysis by phosphorylase, it would seem that within a single fiber both enzymes should have about the same relative activities. If the classic view that glycolysis is a more important metabolic pathway in white than in red fibers is correct, the white fibers should have a higher concentration of enzymes relating directly to glycogen metabolism than do the red fibers. Certainly glycogen levels are higher in white than in red muscle. Histochemical evidence concerning the location of the two enzymes was substantiated by their quantitative determination in homogenates of tissues consisting of predominantly red or white fiber groups (Tables IV, V, VI). Total phosphorylase and phosphorylase *a* of white muscle was about three times that of red muscle while total transglucosylase activity was about 1.4 times higher in red muscle. It is interesting to note that the relative difference in transglucosylase activity between red and white muscle was less than half of the difference found for phosphorylase. This perhaps may explain the failure to obtain as clear cut histochemical differentiation between fibers of high and low transglucosylase activity as found for phosphorylase activity.

The values for total phosphorylase activity found in our experiments (Tables IV and VI) were lower than the levels reported in the literature for rat muscle (4000 ± 900 phosphorylase units[18]; 6000 ± 200[27]). However, loss of total phosphorylase activity can occur by denaturation of the enzyme[36]. This fact, plus variation in strains of animals, muscles analyzed, conditions of sampling and assay techniques possibly explain differences in total phosphorylase values found in the literature and the results of the present experiments.

The percentage values for active phosphorylase of both red and white muscle reported in this thesis (75-78% of the total phosphorylase) are higher than some of the levels reported in the literature for resting muscle (18-19%, mouse muscle[109]; 28%, rat muscle[27]). It is well known that several factors may alter the content of phosphorylase _a_ in muscle, including the action of epinephrine, anoxia, and stimulation[21,18]. Cori and Illingworth[18] reported that freezing muscle increases phosphorylase _a_ because of the contraction associated with muscle chilling. In obtaining muscle samples for phosphorylase assays the animals were decapitated and exsanguinated to facilitate differentiation of red and white muscle fiber groups. During dissection the muscles occasionally twitched or underwent contraction. In addition muscle samples were frozen and stored in the frozen state. Under these conditions it is not surprising to find the content of phosphorylase _a_ at relatively high levels. However, because comparison of phosphorylase activities was made between paired samples of red and white muscle, and the enzyme activities were assayed under similar conditions, it can be concluded that the

observed differences between levels of total and active phosphorylase in red and white muscle were real.

II. In vivo and in vitro Studies

Glycolysis has been defined as the process by which glycogen, glucose and other hexoses are broken down to pyruvic and lactic acids. For years the assumption has been that white muscle derives energy primarily from glycolysis while red muscle obtains it chiefly from citric acid cycle oxidations. The assumption of high glycolytic activity in white muscle was based on two experimental findings; first, that glycogen levels are higher in resting white muscle than in red and, second, that lactate production is higher in resting white muscle than in red. However, no measurements of glycogen turnover were made, and the amount of lactate produced and then oxidized in the citric acid cycle was not determined.

Previous work from our laboratory demonstrated that under hypoxic conditions red muscle has a higher concentration of α-glycerophosphate (α-GP) than does white muscle[98]. α-GP is formed from dihydroxyacetone-PO_4 in the presence of DPNH. Dihydroxyacetone phosphate, therefore, can compete with pyruvate as a hydrogen acceptor to make more DPN available so that glycolysis may proceed under anaerobic conditions and thereby increase the concentration of pyruvate for oxidation in the citric acid cycle. This data is consonant with a higher rate of glycolysis in red than in white muscle. Diaphragm muscle, which has a higher rate of oxidative activity than does red voluntary muscle, produces more α-GP than red voluntary muscle[98].

Previous work from this laboratory showed that lactate production (amount of lactate appearing in the incubation medium) by red muscle was higher than by white during a 15 minute equilibration period (red, 1.26 ± 0.14; white, 0.91 ± 0.07 s.e., mg/g; $n = 6$, $P < .005$ on the basis of paired observations[1]). Prior to incubation for the equilibration period the muscle tissues were subjected to conditions which produced hypoxia within the cells (dissection, weighing, etc.). Under these experimental conditions glycolytic mechanisms should predominate over oxidative processes. Since white muscle is assumed to have a higher rate of anaerobic glycolysis, a higher lactate production by white muscle than by red would be expected. However, since no data was available on glucose uptake, the change in glycogen level, or the amount of lactate produced and then utilized by the citric acid cycle in the two types of muscle during the equilibration period, definite conclusions relative to glycolytic activity cannot be drawn.

Although there is experimental evidence to support the concept that glycolysis is more active in red muscle than in white, as indicated in the two instances cited above, it is not valid to interpret the results of the glucose-C^{14} incorporation studies in this paper as indicative of higher rates of glycogen turnover and glycolysis in red muscle. Several criteria must be satisfied to establish turnover rates: (a) the specific activity of the immediate precursor must be established; (b) conditions of steady state should be met; and (c) the concentration or pool of metabolite must be homogeneous, with newly

[1]Unpublished results

formed molecules being almost instantly diluted and integrated into the whole pool.

With respect to glycogen metabolism, these criteria have not been satisfied. Although the glucose uptake of red and white muscle was the same[5], the per cent of uptake appearing in the glycogen compartment was not the same for the two types of muscle. Furthermore, the pool size and specific activity of UDPG, the immediate precursor of glycogen, was not determined. Steady state is defined as that condition in which there is no net concentration change, with rates of formation and breakdown being equal. Steady state conditions were not present in the in vitro experiments as the glycogen levels were decreasing during the course of incubation (Table XII). In addition, the specific activities of the glycogen fractions as given in figure 9 would seem to indicate that a steady state was not established in the glycogen of either red or white muscle.

Glycogen has been shown to be a non-homogeneous metabolic pool. The molecular weight of glycogen has been found to vary widely depending on conditions of isolation (rat muscle, TCA-extractable glycogen, MW = 43.8×10^6; KOH-soluble glycogen, 6.1×10^6 [116]). Rabbit liver glycogen has been shown to vary in molecular weight from 1×10^6 to 190×10^6 [62]. Stetten and Stetten[116] have demonstrated in vivo that the larger glycogen molecules in muscle are more active in incorporating glucose-C^{14} than are the smaller ones. In addition, glycogen molecules are highly branched structures, and the Stettens have shown that six hours after injection of glucose-C^{14} in an animal the specific activities of the outermost tiers of glucosyl residues are 1-1/2

times that of the total glycogen. Within 48 hours, the specific activity of the interior portion of the glycogen molecules (limit dextrin) increased indicating a gradual incorporation of outer portions of the molecule into the interior of the glycogen structure. Therefore, the probability of turnover of a glucosyl residue in a given time would depend on the size of the glycogen molecule to which it is attached and also on its location in the glycogen molecule, whether on the outer branches or more centrally located. It is apparent, then, that the glycogen pool is non-homogeneous and that absolute rates at which new glucosyl units are added and removed cannot be determined with certainty. For these reasons, the term 'turnover rate' with reference to the labeling of glycogen has been avoided in this discussion.

Another factor which tends to affect the homogeneity of the glycogen pool relates to the controversy surrounding the division of tissue glycogen into two forms. One fraction, readily extractable in cold trichloroacetic acid (TCA), has been termed the TCA-soluble, free, or lyo-glycogen. A second fraction, remaining after mild extraction procedures, is termed residual, fixed, or desmo-glycogen. Metabolically, the amount of glycogen and the ratio of free to fixed glycogen in various tissues is sensitive to various endocrinological, nutritional and other conditions[115]. The validity of the division of glycogen into two fractions has been questioned by several investigators including Roe *et al*[106] and Hanson *et al*[40]. The question of which glycogen fraction is metabolically more active has also been disputed. Russell and Bloom[110] suggested that since TCA-extractable glycogen is more labile under certain physiological conditions, this

fraction is the more active metabolically than residual glycogen. Stetten et al[117], however, showed in vivo that the administration of glucose-C^{14} resulted in a higher specific activity in residual glycogen than in the extractable fraction. Kits van Heijningen[51] investigated the labeling of isolated rat diaphragm glycogen and found, in agreement with Stetten, that after one and two minutes incubation residual glycogen had a higher specific activity than did the extractable glycogen (two and three observations).

III. Specific Activities of Glycogen Fractions, Red versus White Muscle

In the present studies in which the glycogen was labeled with C^{14}, the specific activity of the TCA-soluble glycogen was higher than that of the residual fraction isolated from red muscle (in vitro experiments, Table VIII). However, in vivo, the specific activities of both fractions in red muscle were similar. The difference in C^{14} incorporation into the TCA-soluble glycogen fraction of red muscle in vitro and in vivo may have been due to various physiological factors which were inadequately controlled in the in vivo preparations. Effects of circulating hormones, anoxia, regulation of blood flow, and stress of the biopsy procedure all may alter rates of carbohydrate metabolism in muscle. Although the C^{14} activity present in the blood of the animals was not determined at the time of biopsy (15-30 minutes), it was found that 45 minutes after injection of 50 μc of glucose-C^{14} the activity of the blood was approximately 1.8×10^6 dpm/ml of blood. Ninety minutes after administration of the label the values had decreased to 6.7×10^5 dpm/ml, indicating that active

uptake by tissues was proceeding beyond the time of muscle biopsy. Stetten et al have estimated the circulation half-life of glucose as approximately one hour[114]. It would appear, therefore, that availability of glucose-C^{14} was not a factor in the in vivo experiments.

IV. Specific Activities of Glycogen from Red and White Muscle, TCA-soluble and Residual Fractions

In his study of in vitro incorporation of glucose-C^{14} into glycogen by rat diaphragm Kits van Heijningen[51] found that after one and two minutes incubation the specific activity of the residual fraction was higher than that of the free glycogen. However, from the data presented in his paper, it also appears that after seven and 15 minutes incubation the specific activity of extractable glycogen was higher than that of the fixed (averages differ by more than three times the standard error of the mean). The activities of both glycogen fractions of diaphragm were similar after 45 and 75 minutes incubation. In the present experiments, after 10 minutes incubation, the specific activities of free and fixed glycogen of red muscle were similar and no data is available to ascertain whether, with shorter periods of incubation, the amount of label incorporated into the TCA-soluble glycogen would be higher than that in the residual fraction.

Kits van Heijningen suggested that, in view of the results of the one and two minute experiments, residual glycogen may serve as a precursor of the soluble fraction in diaphragm. From experimental data plotted in figure 9 there is no indication that a product-precursor relationship exists between the TCA-soluble and residual glycogen of red muscle. In the first time period assayed (10 minutes)

there was no statistical significance between the specific activities of residual and TCA-soluble glycogen (Table VIII). It is possible that the incorporation of label into residual glycogen may have been higher than into the free fraction if muscle samples had been assayed after shorter periods of incubation. However, figure 9 demonstrates that in red muscle the amount of label incorporated into residual glycogen approached a constant specific activity while the amount of label in the extractable glycogen was still increasing with time. This may indicate an intrinsic difference in the metabolism of free and fixed glycogen in red muscle. Since in diaphragm the specific activities of free and fixed glycogen were similar after 45 minutes incubation, whereas in red muscle the amount of label appearing in TCA-soluble glycogen was increasing after 120 minutes incubation, these observed differences may demonstrate a variation in glycogen metabolism between red voluntary skeletal muscle and diaphragm. The specific activities for free and fixed glycogen in white muscle were similar for all experimental periods, again demonstrating a possible difference in glycogen metabolism between skeletal muscle and diaphragm.

In vivo, there was no difference in the specific activities of the two glycogen fractions for either red or white muscle. These results do not agree with the observations made by Stetten et al[117] who found that in vivo, after several successive TCA extractions, the residual glycogen had a higher specific activity than the TCA-soluble fraction. The type of muscle used in Stetten's investigations was not specified and the extraction technique differed from that used in

the experiments reported here. Stetten et al also found that glycogen isolated by extracting with cold TCA had a molecular weight about seven times that of glycogen isolated after KOH digestion without prior TCA extraction. Stetten's conclusion[116] was that in muscle larger molecules of glycogen isolated by either method participate more actively in the process of incorporating new glucosyl residues at their periphery than do smaller molecules.

V. Levels of TCA-soluble and Residual Glycogen

Russell and Bloom[110] reported that the free glycogen of gastrocnemius muscle is about 50% of the total glycogen, a figure which agrees with the values found in red muscle in the present study in vitro. In in vitro experiments with predominantly white adductor fibers the levels of residual glycogen were higher than the levels of free in four of the seven experimental periods (Table XII). In vivo, with both red and white muscle, the residual glycogen levels were always higher than the TCA-soluble fraction (Table XIV). Although the levels of glycogen in rat diaphragm after one and two minutes incubation were too variable to be meaningful[51], concentrations of free and fixed glycogen were similar after seven minutes incubation with glucose-C^{14}. However, the levels of free glycogen were greater than those of the fixed fraction after 15, 45 and 75 minutes incubation (means differ by more than 3 times the standard error of the mean).

VI. Significance of Experimental Observations

The specific activity of glycogen is always higher in red muscle than in white, both in vivo and in vitro (Tables VIII and XIII). Red

muscle has a higher rate of oxidative activity than white, indicated by the higher rate of $C^{14}O_2$ production. Since any C^{14} derived from the tricarboxylic acid cycle must have passed through the Embden-Meyerhof pathway, this might imply that the pathway:

$$\text{glucose} \longrightarrow \text{glucose-6-PO}_4 \rightleftarrows \text{pyruvate} \rightleftarrows \text{TCA cycle} \qquad \text{(A)}$$

is more active in red muscle. Since the specific activity of glycogen is higher in red muscle, the pathway:

$$\text{glucose-6-PO}_4 \rightleftarrows \text{glucose-1-PO}_4 \rightleftarrows \text{glycogen} \qquad \text{(B)}$$

also appears to be more active in red muscle.

However, additional factors must be taken into consideration. During the <u>in vitro</u> experimental period, the decrease in total glycogen levels from the controls to the 120 minute samples averaged -0.66 for red and -1.38 for white muscle, which apparently indicates a greater glycolytic activity in white muscle. Since these changes in glycogen levels are also influenced by the amount of glucose incorporated into glycogen during incubation, the net change in glycogen concentration is the algebraic sum of glucose units added and removed during the experimental period. It is possible to approximate the amount of glycogen formed from glucose knowing: (1) the glucose uptake per unit time (mg/g tissue/hour); (2) the per cent of this uptake which appears in glycogen; and (3) the activity of the glycogen in terms of counts per minute per gram of tissue. Previous work in this laboratory has established that the glucose uptake for red and white fibers are similar (1.5 mg/g/hr)[5], and that 15% of the glucose

uptake is recovered in glycogen after one and two hours incubation (50% red and 50% white muscle fibers). With these figures, the per cent of glucose uptake going into glycogen of red and white muscle can be calculated from the activity of glycogen in red and white muscle.

When the amount of glucose converted to glycogen was calculated, it was found that the per cent glucose uptake appearing in the glycogen fraction was 3- to 4-fold higher in red than in white muscle. Therefore, although the decrease in the absolute level of glycogen was less in red than in white muscle, the net change in glycogen was about equal in the two types of muscle. This would indicate, then, that glycolysis is equally active in red and white muscle, at least in the resting state.

Another factor to be considered is the relative activities of the branching and debranching enzymes in the two types of muscle. If the rates of addition and removal of glucosyl units on glycogen molecules are similar for red and white muscle, but the rate of branching by which labeled glucosyl units are incorporated into the interior of glycogen molecules and conserved to give a higher specific activity (possibly coupled with a low debranching enzyme activity) is also higher in red muscle, the increase in the amount of label incorporated into glycogen would not reflect an increased rate of glycogenesis.

Some of the observed differences in specific activity of glycogen from red and white muscle may be reconciled with the results from histochemical and homogenate assays for transglucosylase and phosphorylase activities. If it is assumed that the levels of these enzymes as measured <u>in vitro</u> reflect <u>in vivo</u> activities, red muscle (high

transglucosylase) forms glycogen more readily than white, and, under the conditions of these experiments, probably degrades glycogen at approximately the same rate as white muscle. Conversely, white muscle (lower glycogen synthetic activity) forms glycogen at a slower rate, but, on metabolic demand (severe exercise), potentially can mobilize glycogen at a more rapid rate because of the high phosphorylase activity.

If the classic view is correct and both pathways A and B (page 92) are more active in white muscle, then the low incorporation of labeled glucosyl units into the glycogen of white muscle could be explained in terms of a preferential turnover of the newly added C^{14} units.

It is interesting to speculate whether a predominant species of glycogen is characteristic of red or white muscle in view of the differences observed in rate of labeling with size of the glycogen molecule. Also, the significance of the distribution patterns of glycogen particles observed by electron microscopy in relation to metabolic activity should be determined.

The studies reported in this thesis have presented information on striking differences observed in the metabolic patterns of red and white muscle. It should be mentioned that the experimental conditions of the Warburg apparatus do not approximate <u>in situ</u> conditions but probably simulate to some degree conditions in resting muscle. Muscle, <u>in situ</u>, even at rest, is constantly under a certain degree of tension. Excised fibers of the <u>in vitro</u> system have contracted from the <u>in situ</u> resting length to an equilibrium length assumed when no

tension is placed on the muscle. To what extent the same metabolic patterns, observed *in vitro*, exist in muscle under tension and/or undergoing contraction remains to be determined. However, because of the non-physiological aspects of *in vitro* experiments, it was reassuring to find the over-all results of *in vitro* studies confirmed by *in vivo* experiments.

Analysis of these experimental results has raised the question as to whether glycolysis is equally active in red and white muscle, or perhaps is even more active in red muscle. If the higher specific activity of glycogen reflects a higher rate of glycogen synthesis in red muscle than in white, this higher metabolic activity in the pathway of glucose to glycogen may hold true only under conditions of these experiments in which muscle is in a resting state. Further investigations are planned to resolve the question as to the relative importance of the glycolytic pathway in red and white muscle under various experimental conditions.

SUMMARY AND CONCLUSIONS

1. Glycogen metabolism of red and white muscle has been investigated in muscle from the hind limbs of the rat.

2. Visual differentiation of muscle samples into predominantly red or white fiber groups was verified by examination of frozen sections stained for succinic dehydrogenase and also by extraction of the formazan reaction product formed by homogenates of red and white muscle.

3. In agreement with histochemical evidence, muscle homogenate studies show that uridine diphosphoglucose transglucosylase activity was higher in red muscle and phosphorylase activity was higher in white muscle.

4. Incorporation of glucose-C^{14} into glycogen from red muscle *in vitro* was from 2 to 16 times higher than that from white muscle.

5. In red muscle the specific activity of TCA-soluble glycogen was significantly higher than that of the residual fraction. No differences were found in the specific activities of the two glycogen fractions from white muscle.

6. Control levels of total glycogen were higher in white muscle than in red. During the course of two hours incubation the levels of glycogen of both fractions decreased, but this decrease was greater in white muscle. However, calculation of the amount of glucose converted into glycogen over the experimental period indicates that conversion was higher in red muscle and therefore the total amount of glycogen metabolized was approximately the same for both types of muscle. Although in white muscle the levels of TCA-soluble glycogen

were lower than the levels of the residual fraction, no differences were found in the levels of the two glycogen fractions in red muscle.

7. *In vivo* experiments confirmed *in vitro* observations that the specific activity of glycogen from red muscle was higher than that from white muscle.

8. These results may be interpreted as indicating that in resting muscle glycolysis is of equal magnitude in red and white muscle. This concept is opposed to the classical view of the relative importance of metabolic pathways which considers that glycolytic mechanisms predominate in white muscle and oxidative processes in red muscle.

9. The results obtained demonstrate further striking metabolic differences in the metabolism of red and white muscle.

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REFERENCES

1. Ahlgren, G. Über die spontanreduzierende einwirkung der muskulatur auf methylenblau. Ein beitrag zur physiologie der dehydrogenasen. Skand. Arch. Physiol., 1921. 41, 1-30.

2. Andersson-Cedergren, E., & Muscatello, U. The participation of the sarcotubular system in glycogen metabolism. J. Ultrastruct. Res., 1963. 8, 391-401.

3. Appleman, M. M., Belocopitow, E., & Torres, H. Factors affecting the activity of muscle glycogen synthetase. Biochem. Biophys. Res. Commun., 1964. 14, 550-554.

4. Beatty, C. H., Peterson, R. D., & Bocek, R. M. Effect of insulin on carbohydrate metabolism of skeletal muscle fibers and diaphragm from control and pancreatectomized rats. J. Biol. Chem., 1960. 235, 277-281.

5. Beatty, C. H., Peterson, R. D., & Bocek, R. M. Metabolism of red and white muscle fiber groups. Am. J. Physiol., 1963. 204, 939-942.

6. Beatty, C. H., Peterson, R. D., Bocek, R. M., Craig, N. C., & Weleber, R. Effect of glucagon on incorporation of glycine-C^{14} into protein of voluntary skeletal muscle. Endocrinology, 1963. 73, 721-726.

7. Belocopitow, E. The action of epinephrine on glycogen synthetase. Arch. Biochem., 1961. 93, 457-458.

8. Berne, R. M. Intracellular localization of the skeletal-muscle relaxing factor. Biochem. J., 1962. 83, 364-368.

9. Biörck, G. On myoglobin and its occurrence in man. Acta Med. Scandinav., 1949. Supp. 226, p. 18.

10. Blanchaer, M. C., & Van Wijhe, M. Isozymes of lactic dehydrogenase in skeletal muscle. Am. J. Physiol., 1962. 202, 827-829.

11. Blanchaer, M. C., Van Wijhe, M., & Mozersky, D. The oxidation of lactate and α-glycerophosphate by red and white skeletal muscle: I. Quantitative studies. J. Histochem., 1963. 11, 500-504.

12. Bloom, W., & Fawcett, D. W. A textbook of histology. Philadelphia: W. B. Saunders Company, 1962.

13. Bo, W. J. A histochemical comparison of glycogen synthesis from glucose-1-phosphate and uridinediphosphoglucose in uterine sections. Proc. Soc. Exper. Biol. & Med., 1962. 111, 186-189.

14. Bo, W. J., & Smith, M. S. Comparison of UDPG-glycogen synthetase activity of the tongue and uterus. Proc. Soc. Exper. Biol. & Med., 1963. 113, 812-814.

15. Boerhave, H. Institutions de medecine. I. Paris, 1739. p. 275. Traduites du Latin en Française (cited in Biörck(9)).

16. Bullard, H. H. On the interstitial granules and fat droplets of striated muscle. Am. J. Anat., 1912. 14, 1-46.

17. Cori, G. T.,& Green, A. A. Crystalline muscle phosphorylase. II. Prosthetic group. J. Biol. Chem., 1943. 151, 31-38.

18. Cori, G. T., & Illingworth, B. The effect of epinephrine and other glycogenolytic agents on the phosphorylase a content of muscle. Biochim. et Biophys. Acta, 1956. 21, 105-110.

19. Cori, G. T., Illingworth, B., & Keller, P. Muscle phosphorylase. In Colowick, S. P., & Kaplan, N. O. (Ed.) Methods in enzymology. Vol. 1. New York: Academic Press, 1955. pp. 200-205.

20. Cori, G. T., & Larner, J. Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. Biol. Chem., 1951. 188, 17-29.

21. Danforth, W. H., Helmreich, E., & Cori, C. F. The effect of contraction and of epinephrine on the phosphorylase activity of frog sartorius muscle. Proc. Natl. Acad. Sci., 1962. 48, 1191-1199.

22. Davey, C. L. The significance of carnosine and anserine in striated skeletal muscle. Arch. Biochem., 1960. 89, 303-308.

23. Davies, R. E. A molecular theory of muscle contraction: calcium-dependent contractions with hydrogen bond formation plus ATP-dependent extensions of part of the myosin-actin cross-bridges. Nature, London, 1963. 199, 1068-1074.

24. Dubowitz, V., & Pearse, A. G. E. A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. Histochemie, 1960. 2, 105-117.

25. Dubowitz, V., & Pearse, A. G. E. Oxidative enzymes and phosphorylase in central core disease of muscle. Lancet, 1960. II, 23-24.

26. Dubowitz, V., & Pearse, A. G. E. Reciprocal relationships of phosphorylase and oxidative enzymes in skeletal muscle. Nature, London, 1960. 185, 701-702.

27. Eisenstein, A. The effect of pyridoxine deficiency on liver and muscle phosphorylase. Biochim. et Biophys. Acta, 1962. 58, 244-247.

28. Embden, G., & Lawaczeck, H. Uber den cholesteringehalt verschiedener kaninchen muskeln. Ztschr. Physiol. Chem., 1923. 125, 199-209.

29. Engel, W. K. The essentiality of histochemical and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. Neurology, 1962. 12, 778-794.

30. Friedman, D. L., & Larner, J. Studies on UDPG-α-glucan transglucosylase. III. Interconversion of two forms of muscle UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochemistry, 1963. 2, 669-675.

31. George, J. C., & Naik, R. M. Studies on the structure and physiology of the flight muscles of bats: 1. The occurrence of two types of fibers in the pectoralis major muscle of the bat (Hipposideros speoris), their relative distribution, nature of the fuel store and mitochondrial content. J. Animal Morph. & Physiol., 1957. 4, 96-101.

32. George, J. C., & Scaria, K. S. Histochemical demonstration of lipase activity in the pectoralis major muscle of the pigeon. Nature, London, 1958. 181, 783.

33. George, J. C., & Talesara, C. L. Studies on the structure and physiology of the flight muscles of birds. 9. Quantitative study of the distribution pattern of succinic dehydrogenase in the pectoralis major muscle of the pigeon. Biol. Bull., 1960. 118, 262-268.

34. George, J. C., & Talesara, C. L. Histochemical observations on the succinic dehydrogenase and cytochrome oxidase activity in pigeon breast-muscle. Quart. J. Microsc. Sci., 1961. 102, pt. 1, 131-141.

35. George, J. C., & Talesara, C. L. Histochemical demonstration of certain DPN-linked dehydrogenases and of aldolase in the red and white fibres of pigeon breast-muscle. Quart. J. Microsc. Sci., 1962. 103, pt. 1, 41-46.

36. Godlewski, H. G. Are active and inactive phosphorylases histochemically distinguishable? J. Histochem., 1963. 11, 108-112.

37. Gomori, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. & Clin. Med., 1942. 27, 955-958.

38. Green, A. A., & Cori, G. T. Crystalline muscle phosphorylase. I. Preparation, properties and molecular weight. J. Biol. Chem., 1943. 151, 21-29.

39. Grűtzner, P. Zur anatomie und physiologie der quergestreiften muskeln. Tome 1. Recueil Zoolog, Suisse, 1884 (cited in Bullard[16]).

40. Hanson, R. W., Schwartz, H. S., & Barker, S. B. "Free" and "fixed" glycogen as physiological entities. Am. J. Physiol., 1960. 198, 800-806.

41. Helmreich, E., & Cori, C. F. The role of adenylic acid in the activation of phosphorylase. Proc. Natl. Acad. Sci., 1964. 51, 131-138.

42. Hess, R., & Pearse, A. G. E. Dissociation of uridine diphosphate glucose-glycogen transglucosylase from phosphorylase activity in individual muscle fibers. Proc. Soc. Exper. Biol. & Med., 1961. 107, 569-571.

43. Hildebrandt, G. F. Lehrbuch der anatomie des menschen II. Braunschweig, 1789 (cited in Biorck[9]).

44. Himmelhoch, S. R., & Karnovsky, M. J. The histochemical demonstration of glyceraldehyde-3-PO_4 dehydrogenase activity. J. Biophys. Biochem. Cytol., 1961. 9, 573-581.

45. Horowitz, F., & Hardin, R. L. In Neurath, H., & Bailey, K. (Ed.) The proteins. Vol. II. New York: Academic Press, 1954. p. 323.

46. Hughes, R. C., Yunis, A. A., Krebs, E. G., & Fischer, E. H. Comparative studies on glycogen phosphorylase. III. The phosphorylated site in human muscle phosphorylase a. J. Biol. Chem., 1962. 237, 40-43.

47. Huls, H. N., & Leonard, S. L. Phosphorylase activity in denervated skeletal muscle. Proc. Soc. Exper. Biol. & Med., 1961. 108, 224-228.

48. Huxley, A. F. Local activation of muscle. Ann. N. Y. Acad. Sci., 1959. 81, 446-452.

49. Huxley, H. E. Muscle cells. In Brachet, J., & Mirskey, A. E. (Ed.) The cell. Vol. 4, part 1. New York/London: Academic Press, 1960. pp. 365-481.

50. Kare, M. R. Phosphocreatine in red and white muscle. Proc. Soc. Exper. Biol. & Med., 1951. 77, 692-694.

51. Kits van Heijningen, A. J. M. The influence of insulin and adrenaline on the incorporation of C^{14}-labeled glucose into glycogen fractions of the isolated rat diaphragm. Arch. Biochem., 1963. 102, 456-463.

52. Knoll, P. Über helle und trοübe, weisse und rothe queregestreifte muskulatur. Sitz. d. Kais. Akad. d. Wiss. Wien, 1889. Bd. 98 (cited in Bullard(16)).

53. Knoll, P. Über protoplasmaarme und protoplasmareiche muskulatur. Denkschr. d. Kais. Akad. d. Wiss. Wien, 1891. Bd. 98 (cited in Bullard(16)).

54. Kolliker, A. Mickroskopische anatomie II:1. Leipzig, 1850 (cited in Biörck(9)).

55. Krebs, E. G., Kent, A. B., & Fischer, E. H. The muscle phosphorylase b kinase reaction. J. Biol. Chem., 1958. 231, 73-83.

56. Krebs, H. A., & Johnson, W. A. The role of citric acid in intermediate metabolism in animal tissue. Enzymologia, 1937. 4, 148-156.

57. Krebs, H. A., & Lowenstein, J. M. The tricarboxylic acid cycle. In Greenberg, D. M. (Ed.) Metabolic pathways. Vol. 1. New York and London: Academic Press, 1960. pp. 129-203.

58. Krishnan, P. S., & Nelson, W. L. Some observations on the isolation of ATP from skeletal muscle. Arch. Biochem., 1948. 19, 65-73.

59. Kühne, W. Ueber den farbstoff der muskeln. Virchows Arch., 1865. 33, 79-94.

60. Lankester, E. R. Ueber das vorkommen von haemoglobin in den muskeln der mollusken und die verbreitung desselben in den lebendigen organismen. Pflüger's Arch., 1871. 4, 315-320.

61. Larner, J. The action of branching enzymes on outer chains of glycogen. J. Biol. Chem., 1953. 202, 491-503.

62. Larner, J. Genetic and hormonal control of glycogen metabolism. Fed. Proc., 1960. 19, 971-976.

63. Larner, J., & Villar-Palasi, C. Enzymes in a glycogen storage myopathy. Proc. Natl. Acad. Sci., 1959. 45, 1234-1235.

64. Larner, J., Villar-Palasi, C., & Richman, D. J. Insulin-stimulated glycogen formation in rat diaphragm. Ann. N. Y. Acad. Sci., 1959. 82, 345-353.

65. Lawrie, R. A. Biochemical differences between red and white muscle. Nature, London, 1952. 170, 122-123.

66. Lawrie, R. A. The activity of the cytochrome system in muscle and its relation to myoglobin. Biochem. J., 1953. 55, 298-304.

67. Leloir, L. F., & Cardini, C. E. Biosynthesis of glycogen from uridine diphosphate glucose. J. Am. Chem. Soc, 1957. 79, 6340-6341.

68. Leloir, L. F., & Goldemberg, S. H. Synthesis of glycogen from uridine diphosphate glucose in liver. J. Biol. Chem., 1960. 235, 919-923.

69. Lohmann, K. Über das vorkommen und den umsatz von pyrophosphate in zellen. II. Mitteilung: die menge der leicht hydrolysierbaren P-verbindung in tierischen und pflanzlichen zellen. Biochem. Ztschr., 1928. 203, 164-171.

70. Lohmann, K. Über die enzymatische aufspaltung der kreatinephosphosaure; zugleich ein beitrag zum chemismus der muskelkontraktion. Biochem. Ztschr., 1934. 271, 264-277.

71. Lundsgaard, E. Untersuchungen über muskel kontraktionen ohne milchsaurebildung. Biochem. Ztschr., 1930. 217, 162-177.

72. Madsen, N. B. Inhibition of glycogen phosphorylase by uridine diphosphate glucose. Biochem. Biophys. Res. Commun., 1961. 6, 310-313.

73. Mansour, T. E., & Mansour, J. M. Effects of serotonin (5-hydroxytryptamine) and adenosine 3',5'-phosphate on phosphofructokinase from the liver fluke Fasciola hepatican. J. Biol. Chem., 1962. 237, 629-634.

74. Margreth, A. The sarcotubular system and its relation to the control of glycolysis. Biochim. et Biophys. Acta, 1963. 77, 337-339.

75. Markert, C. L., & Ursprung, H. The ontogeny of isozyme patterns of lactic dehydrogenase in the mouse. Dev. Biol., 1962. 5, 363-381.

76. Meier, R., & Meyerhof, O. Die verbrennungswärme des glykogens. Biochem. Ztschr., 1924. 150, 233-242.

77. Meyerhof, O. The chemistry of muscular contraction. Lancet, 1930. 1415-1422.

78. Meyerhof, O. Oxidoreductions in carbohydrate breakdown. Biol. Symposia, 1941. 5, 141-156.

79. Miller, W. L., & Vander Wende, C. UDPG-glycogen transglucosylase of the human leukocyte. Biochim. et Biophys. Acta, 1963. 77, 494-495.

80. Muscatello, U., Andersson-Cedergren, E., Azzone, G. F., & von der Decken, A. The sarcotubular system of frog skeletal muscle. A morphological and biochemical study. J. Biophys. Biochem. Cytol., 1961. 10 (suppl.), 201-218.

81. Nachlas, M. M., Tsou, K., DeSouza, E., Cheng, C., & Seligman, A. M. Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem., 1957. 5, 420-436.

82. Nachmias, V. T., & Padykula, H. A. A histochemical study of normal and denervated red and white muscles of the rat. J. Biophys. Biochem. Cytol., 1958. 4, 47-54.

83. Needham, D. M. Red and white muscle. Physiol. Rev., 1926. 6, 1-27.

84. Newsholme, E. A., & Randle, P. J. Regulation of glucose uptake by muscle. V. Effect of anoxia, insulin, adrenalin and prolonged starving on concentration of hexose phosphates in isolated rat diaphragm and isolated rat heart. Biochem. J., 1961. 80, 655-662.

85. Niemeyer, H. Metabolismo de los hidratos de carbono en el higado. 1955. Cited in DeDuve, C., & Hers, H. G. Ann. Rev. Biochem., 1957. 26, 168.

86. Niemeyer, H., Gonzales, C., & Rozzi, R. The influence of diet on liver phosphorylase. J. Biol. Chem., 1961. 236, 610-613.

87. Ogata, T. A histochemical study of the red and white muscle fibers. Part II. Activity of the cytochrome oxidase in muscle fibers. Acta Med. Okayama, 1958. 12, 228-232.

88. Ogata, T. The differences in some labile constituents and some enzymatic activities between the red and the white muscle. J. Biochem., 1960. 47, 726-732.

89. Padykula, H. A. The localization of succinic dehydrogenase in tissue sections of the rat. Am. J. Anat., 1952. 91, 107-132.

90. Padykula, H. A., & Gauthier, G. F. Cytochemical studies of adenosine triphosphatase in skeletal muscle fibers. J. Cell Biol., 1963. 18, 87-108.

91. Parnas, J. K. The chemistry of muscle. Ann. Rev. Biochem., 1932. 1, 431-456.

92. Parnas, J. K., & Wagner, R. Uber den kohlenhydratumsatz isolierter amphibien muskeln und uber die beziehungen zwichen kohlenhydratschwund und milchsäurebildung im muskel. Biochem. Ztschr., 1914. 61, 387-427.

93. Paul, M. H., & Sperling, E. Cyclophorase system. XXIII. Correlation of cyclophorase activity and mitochondrial density in striated muscle. Proc. Soc. Exper. Biol. & Med., 1952. 79, 352-354.

94. Pearse, A. G. E. Direct relationship of phosphorylase and mitochondrial α-glycerophosphate dehydrogenase activity in skeletal muscle. Nature, London, 1961. 191, 504.

95. Pearse, A. G. E. Histochemistry, theoretical and applied. (2nd Ed.) Boston: Little, Brown & Co., 1961. p. 506.

96. Pearse, A. G. E., & Scarpelli, D. G. Intramitochondrial localization of oxidative enzyme systems. Exper. Cell Res., 1959. 7 (suppl.), 50-64.

97. Pellegrino, C., & Franzini, C. An electron microscope study of denervation atrophy in red and white skeletal muscle fibers. J. Cell Biol., 1963. 17, 327-349.

98. Peterson, R. D., Gaudin, D., Bocek, R. M., & Beatty, C. H. α-glycerophosphate metabolism in muscle under aerobic and hypoxic conditions. Am. J. Physiol., 1964. 206, 599-602.

99. Porter, K. R., & Palade, G. E. Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. J. Biophys. Biochem. Cytol., 1957. 3, 269-300.

100. Rall, T. W., & Sutherland, E. W. II. The enzymically catalyzed formation of adenosine 3'5'-phosphate and inorganic pyrophosphate from adenosine triphosphate (ATP). J. Biol. Chem., 1962. 237, 1228-1232.

101. Ranvier, L. Compt. Rend. Acad. Sci., 1873. 77, 1030 (cited in Needham(83)).

102. Retzius, G. Muskelfibrille und sarcoplasma. Biol. Untersuch., N.F. I, 1890. 1, 51-70.

103. Riesser, O. Beiträge zur physiologie des kreatins. Ztschr. Physiol. Chem., 1922. 120, 187-206.

104. Robbins, P. W., Traut, R. R., & Lipman, F. Glycogen synthesis from glucose, glucose-6-PO_4 and uridine-di-PO_4 glucose in muscle preparations. Proc. Natl. Acad. Sci., 1959. 45, 6-12.

105. Roe, J. H. The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem., 1955. 212, 335-343.

106. Roe, J. H., Bailey, J. M., Gray, R. R., & Robinson, J. N. Complete removal of glycogen from tissues by extraction with cold trichloroacetic acid solution. J. Biol. Chem., 1961. 236, 1244-1246.

107. Rollett. Sitzb. d. k. Ak. Wien, Math-Natwiss. Kl., 1884. 89 (3). (Cited in Needham(83)).

108. Rudolph, G. Der histochemische nachweis dehydrogenasehaltiger sarkosomen (mitochondrien) in der herz-, skelt - und glatten muskulatur (untersuchungen mit den fermenten DPN-diaphorase, TPN-diaphorase und succino dehydrogenase). Acta Histochem., 1961. 12, 48-68.

109. Rulon, R. R., Schottelius, D. D., & Schottelius, B. A. Effect of stimmulation on phosphorylase levels of excised anterior tibial muscles of the mouse. Am. J. Physiol., 1961. 200, 1236-1238.

110. Russell, J. A., & Bloom, W. L. Extractable and residual glycogen in tissues of the rat. Am. J. Physiol., 1955. 183, 345-355.

111. Schmid, R., Robbins, P. W., & Traut, R. R. Glycogen synthesis in muscle lacking phosphorylase. Proc. Natl. Acad. Sci., 1959. 45, 1236-1240.

112. Selinger, Z., & Schramm, M. An insoluble complex formed by the interaction of muscle phosphorylase with glycogen. Biochem. Biophys. Res. Commun., 1963. 12, 208-214.

113. Stein, J. M., & Padykula, H. A. Histochemical classification of individual skeletal muscle fibers of the rat. Am. J. Anat., 1962. 110, 103-123.

114. Stetten, D., & Stetten, M. R. Homopolysaccharides. In Springer, G. F. (Ed.) Polysaccharides in biology--transactions of the third conference. New York: Josiah Macy, Jr. Foundation, 1957. pp. 9-153.

115. Stetten, D., Jr., & Stetten, M. R. Glycogen metabolism. Physiol. Rev., 1960. 40, 505-537.

116. Stetten, M. R., Katzen, H. M., & Stetten, D. Metabolic inhomogeneity of glycogen as a function of molecular weight. J. Biol. Chem., 1956. 222, 587-599.

117. Stetten, M. R., Katzen, H. M., & Stetten, D., Jr. A comparison of the glycogen isolated by acid and alkaline procedures. J. Biol. Chem., 1958. 232, 475-488.

118. Takeuchi, T., & Glenner, G. G. Histochemical demonstration of a pathway for polysaccharide synthesis from uridine diphosphoglucose. J. Histochem., 1960. 8, 227-230.

119. Takeuchi, T., & Kuriaki, H. Histochemical detection of phosphorylase in animal tissue. J. Histochem., 1955. 3, 153-160.

120. Takeuchi, T., Tadokoro, N., & Ide, H. Histochemical studies on glycogen synthesis from glucose-1-PO_4 in blood cells under normal and pathological conditions. J. Histochem., 1962. 10, 572-579.

121. Umbreit, W. W., Burris, R. H., & Stauffer, J. F. Manometric techniques. (3rd Ed.) Minneapolis, Minn.: Burgess Publishing Co., 1957.

122. Van Wijhe, M., Blanchaer, M. C., & Jacyk, W. R. The oxidation of lactate and α-glycerophosphate by red and white skeletal muscle: II. Histochemical studies. J. Histochem., 1963. 11, 505-510.

123. Villar-Palasi, C., & Larner, J. Levels of activity of the enzymes of the glycogen cycle in rat tissues. Arch. Biochem., 1960. 86, 270-273.

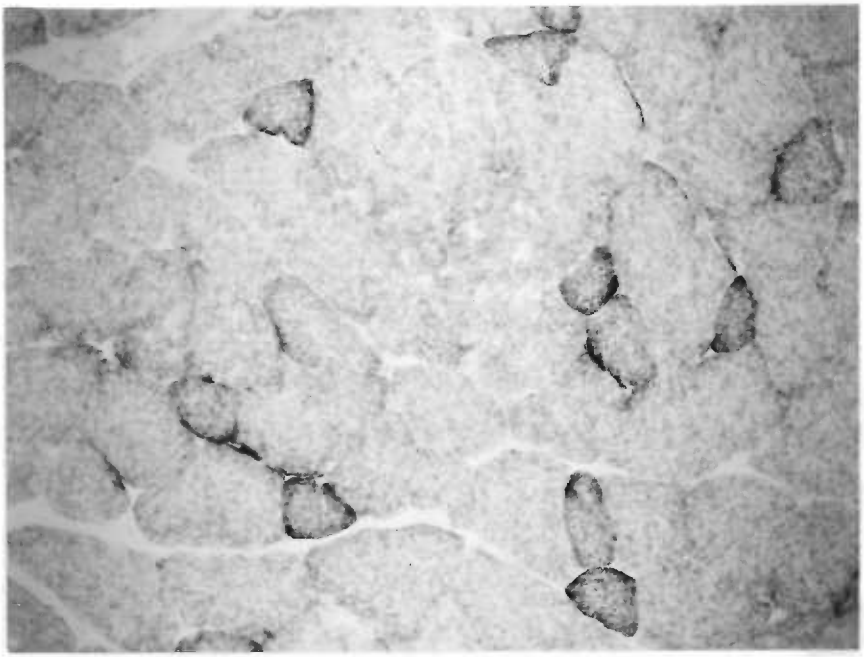
124. Weichselbaum, T. E. An accurate and rapid method for the determination of proteins in small amounts of blood, serum and plasma. Am. J. Clin. Path., Tech. Suppl., 1946. 10, 40-49.

Plate I a. Section of muscle fiber groups from the white area of semimembranosus muscle, stained for succinic dehydrogenase (Nitro-BT). The smaller, darker staining fibers contrast with the larger, more pale staining fibers and make up less than 25% of the total area represented. This section is classified as 1+ for histochemical evaluation of the degree of "redness".

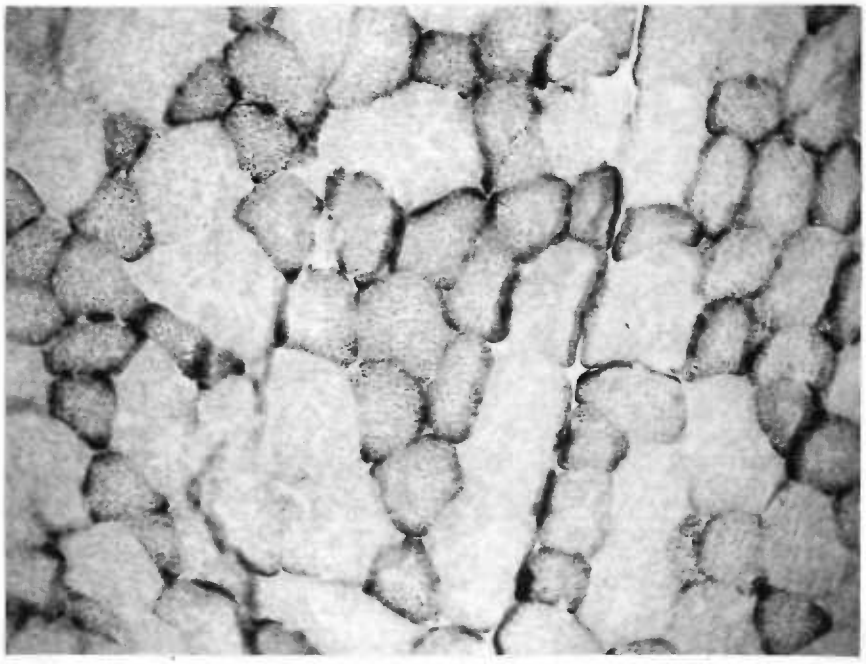
x 250.

Plate I b. Section of muscle fibers from the red area of the semimembranosus muscle (succinic dehydrogenase). The intrafibrillar spaces are more deeply stained in the small red fibers but appear to have a beaded appearance in both large and small fibers. Histochemical classification, 3+.

x 250.



Ia.



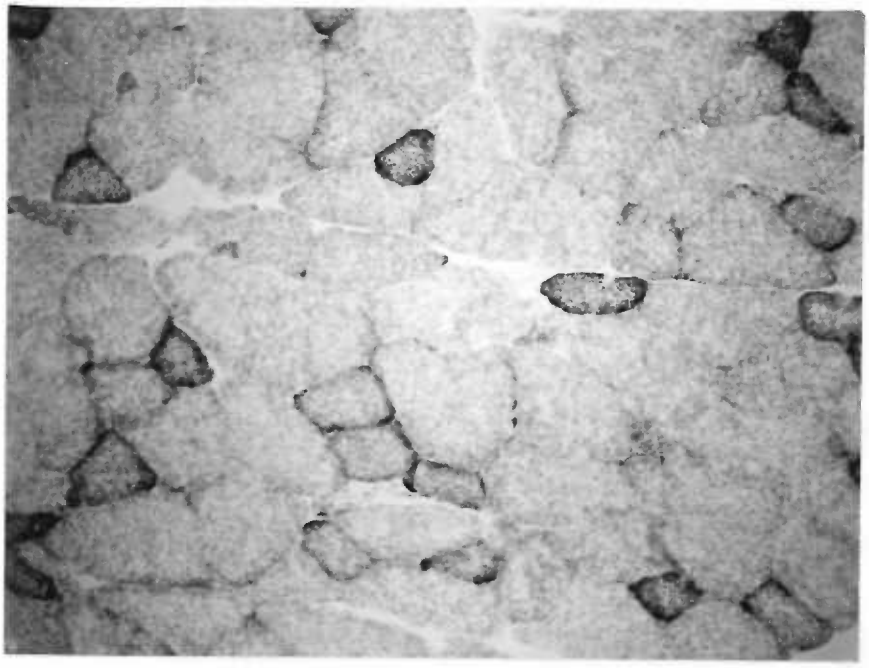
Ib.

Plate II a. Section of muscle fibers from the white area of the semitendinosus muscle (succinic dehydrogenase). The appearance is very similar to that of the white muscle of the semimembranosus. Classification, 1+.

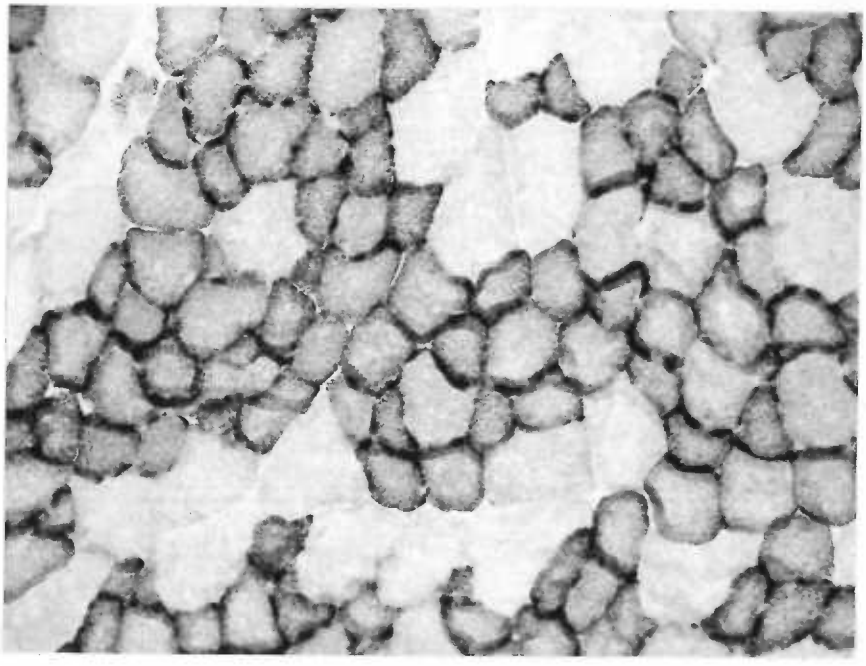
x 250.

Plate II b. Section of muscle fibers from the red area of the semitendinosus muscle (succinic dehydrogenase). In addition to the large, pale and small dark fibers, fibers of intermediary size and color are noted. Histochemical classification, 4+.

x 250.



II a.



II b.

Plate III a. Section of the caudofemoralis muscle (succinic dehydrogenase). This muscle consists principally of red fibers throughout. Many of the small, dark fibers have heavy deposits of formazan immediately beneath the sarcolemma. These deposits correspond to the site of the giant mitochondria previously described[90]. Excellent examples of dark staining areas near the sarcolemma are found also in Plates I b, II b, and III c. Classification, 3+.

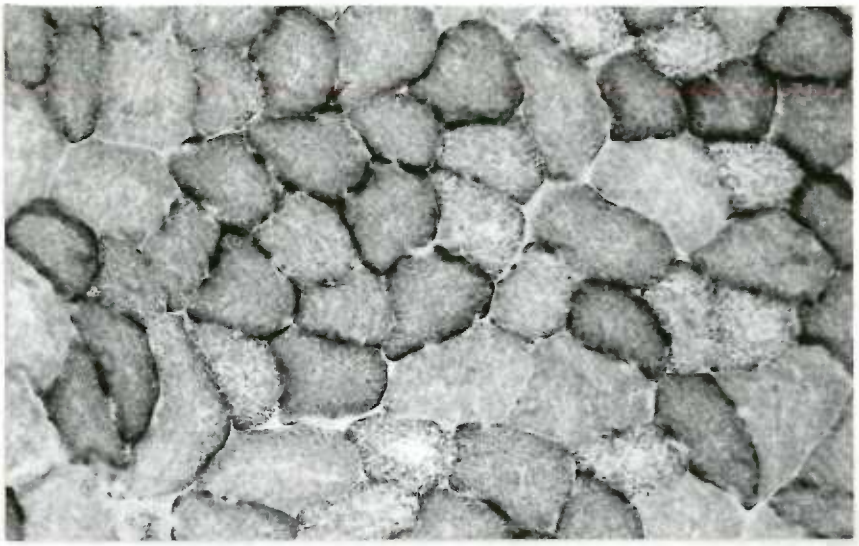
x 250.

Plate III b. Section of muscle fibers from the white area of the gastrocnemius muscle. Three types of fibers are found. Interfibrillar deposits of formazan are sharply defined in all three types of fibers. The spaces occupied by the contractile material appear much larger in the pale fibers than in darker fibers. Classification, 1+.

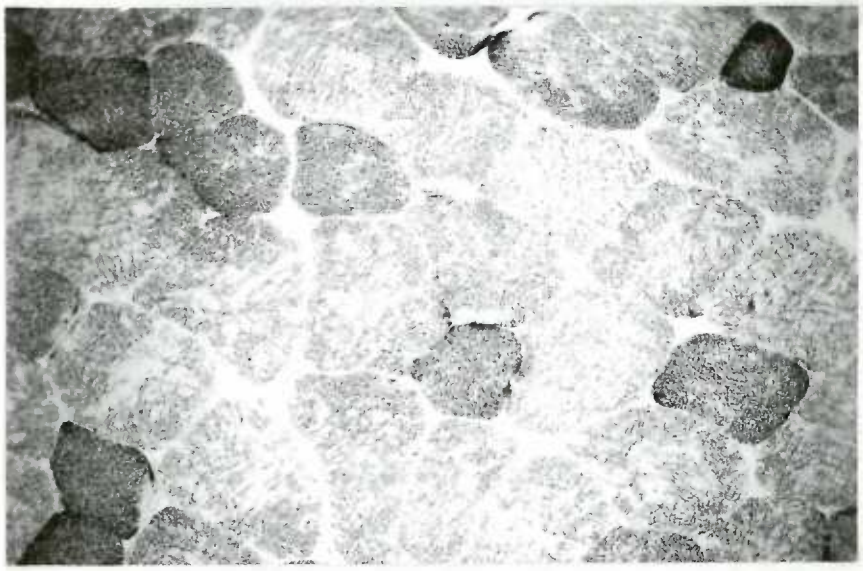
x 250.

Plate III c. Section of soleus muscle (succinic dehydrogenase). Muscle fibers are more uniform in size and staining than in examples of other muscles shown, although some fibers appear darker than others. Classification, 4+.

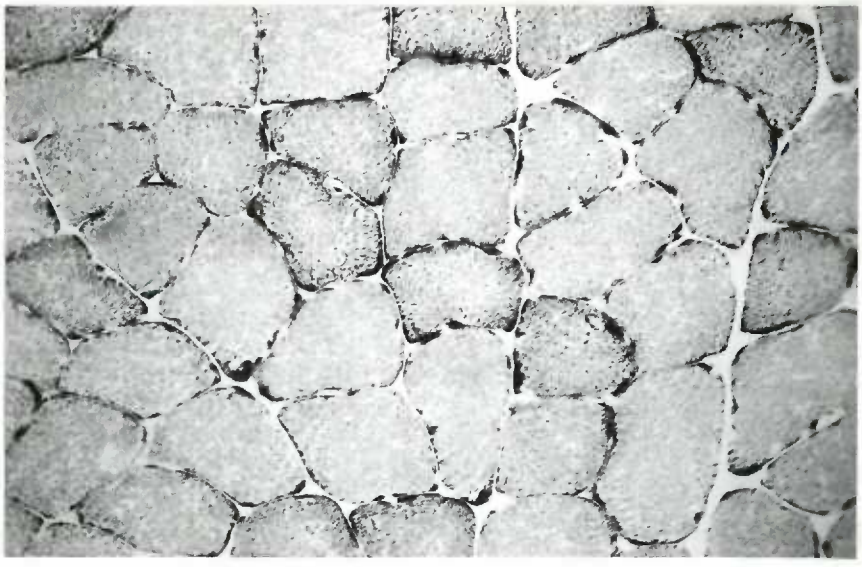
x 250.



III a.



III b.



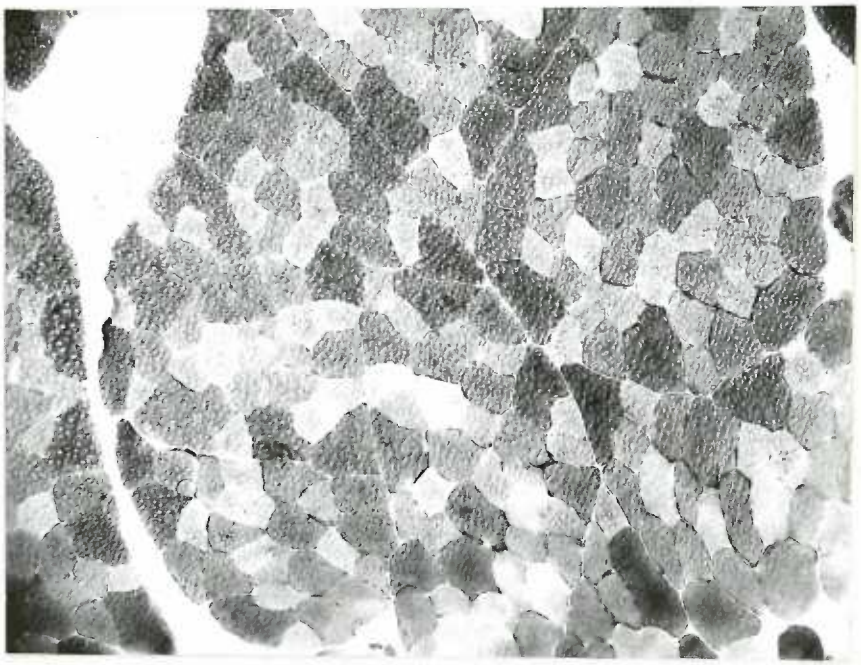
III c.

Plate IV a. Section of the red area of the semimembranosus muscle stained for phosphorylase. In general, larger fibers are more deeply stained than are the smaller fibers. Fibers of an intermediary degree of staining are also seen. This is a serial section to IV b.

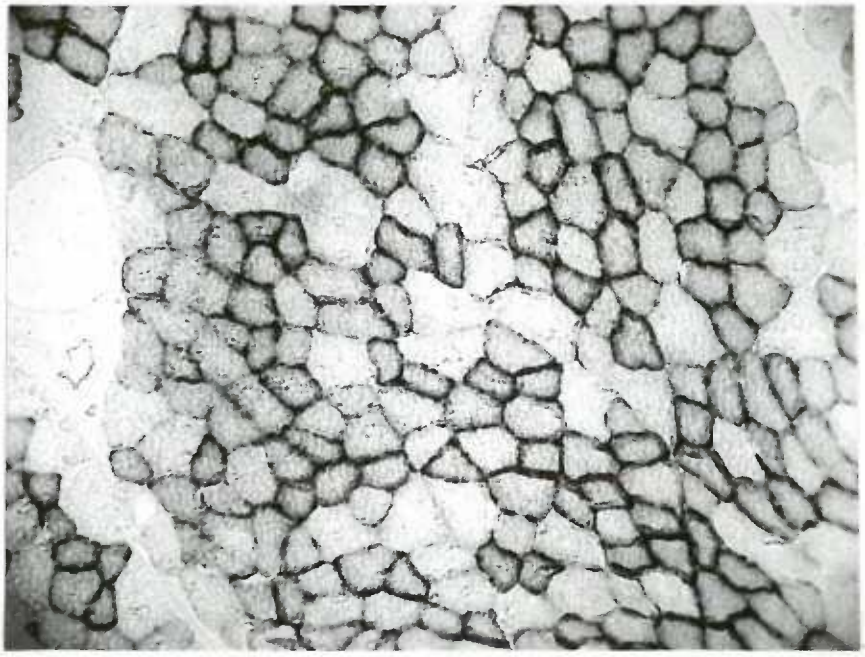
x 100.

Plate IV b. Serial section to IV a stained for succinic dehydrogenase. Large fibers stain lightly and smaller fibers are darker, demonstrating that fibers high in oxidative enzymes are low in phosphorylase activity.

x 100.



IV a.



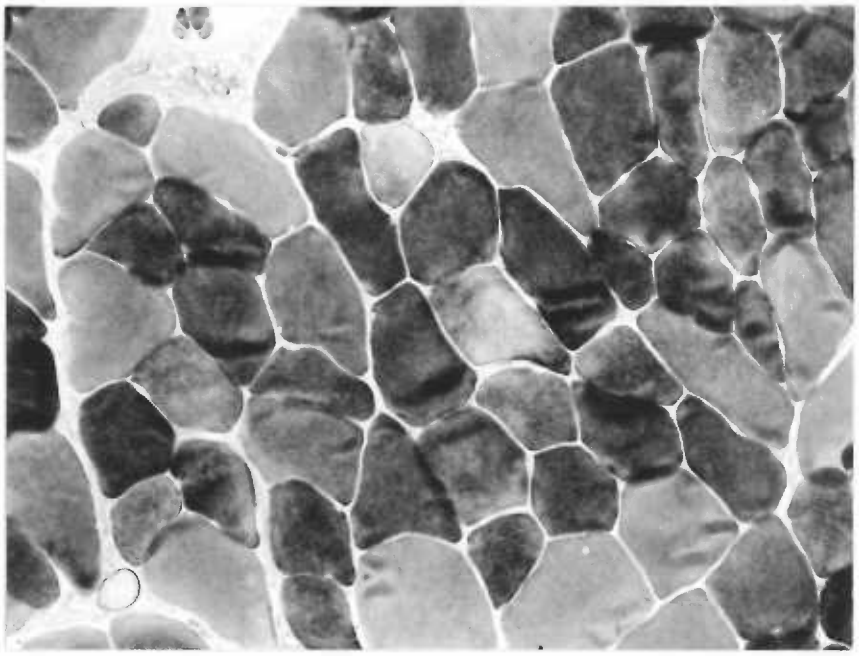
IV b.

Plate V a. Section of caudofemoralis muscle stained for transglucosylase activity and serial to V b. Comparison of the two sections shows that fibers high in transglucosylase activity are also high in succinic dehydrogenase activity.

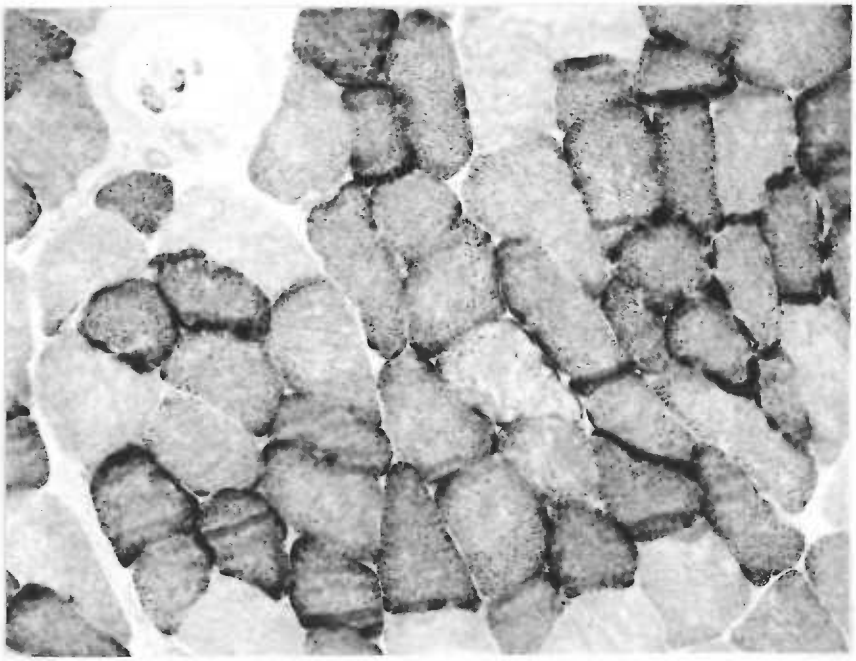
x 250.

Plate V b. Section of caudofemoralis muscle stained for succinic dehydrogenase and serial to V a.

x 250.



V a.



V b.

Plate VI a. Section of caudofemoralis muscle stained for transglucosylase and serial to VI b and VI c. Comparison of the three serial sections indicates that the fibers high in transglucosylase activity are also high in succinic dehydrogenase and low in phosphorylase activities.

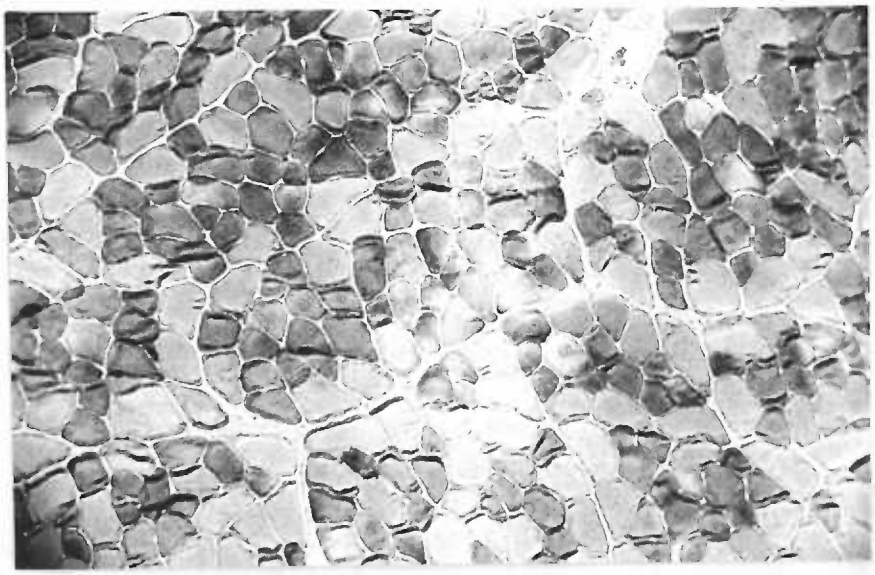
x 100.

Plate VI b. Caudofemoralis muscle stained for succinic dehydrogenase and serial to VI a and VI c.

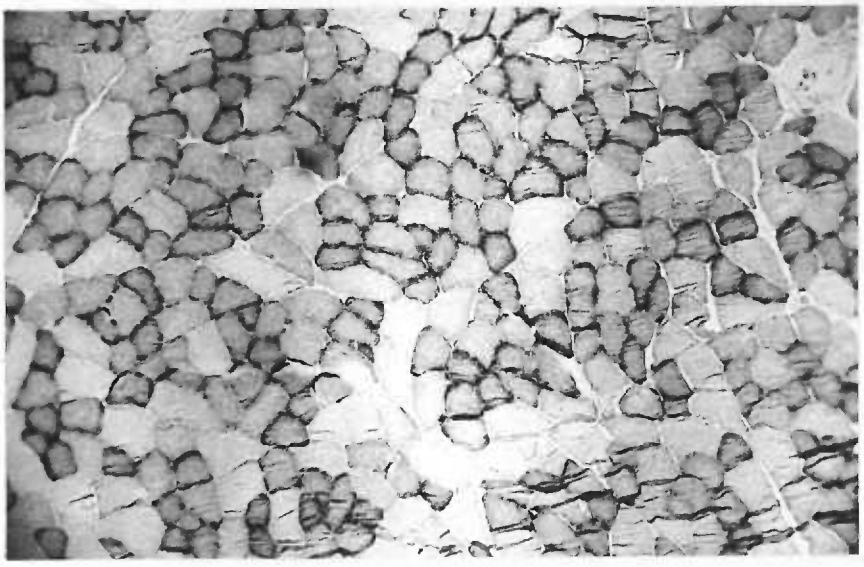
x 100.

Plate VI c. Caudofemoralis muscle stained for phosphorylase activity and serial to VI a and VI b.

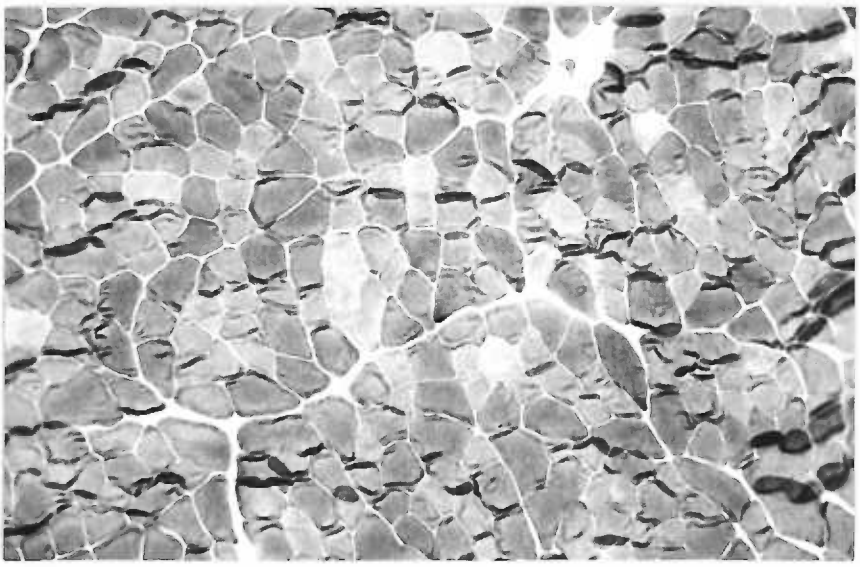
x 100.



VI a.



VI b.



VI c.