STUDIES CONCERNING THE EFFECT OF INSULIN ON CARBOHYDRATE METABOLISM OF RED AND WHITE MUSCLE

by

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I. Introduction

A. Properties of Voluntary Skeletal Muscle

1. Functional

Voluntary skeletal muscle is designed to perform under a wide variety of conditions. The two functions of muscle are to perform work and to generate force in the form of tension. Muscle contraction is isotonic or isometric. Like other organs, voluntary skeletal muscle is well adjusted to its functions. A. V. Hill (69) points out that muscles are designed to provide a compromise between various needs. There must be a balance between speed of movement and economy of energy in maintaining force or posture. For example, the ocular muscles, which cause rapid movements of the eye, contract rapidly, while the soleus muscle, which supports the body against gravity, contracts slowly. Furthermore, every muscle has two optimum speeds of shortening. One speed allows maximum efficiency while the other permits maximum power. Thus, the intrinsic speed of muscles varies, and in any study of the function of voluntary skeletal muscle this property must be considered.

2. Structural

The striated appearance of voluntary skeletal muscle was established during the 19th century, chiefly by the use of direct light microscopy. Bowman (1840) observed that each muscle fiber consists of a mass of fibrils which lie parallel to the longitudinal axis of the fiber, and that the whole fiber is surrounded by a sarcolemma (23). He suggested that striations in resting muscle fibers are

visible because different bands possess different refractive indexes. A broad band of higher refractive index is optically anisotropic (A band) while a band of lower refractive index is optically isotropic (I band). Dobie (38) also noted a narrow line of high refractive index (Z line). With the development of better light microscopy, A, I, and H bands as well as the Z line were further characterized. Studies of striated muscle fiber by use of the electron microscope have provided valuable information concerning the ultrastructure of myofibrils (63). These studies have demonstrated that structural proteins are organized into longitudinal myofilaments. Striation of the myofibrils is due to special organization of the myofilaments at the molecular level. Huxley (72) demonstrated that muscle contains two separate structural proteins, actin and myosin, which are organized into separate filaments. The thicker filaments contain myosin and are found only in the A band, while the thinner filaments contain actin and are found in all bands except in the H zone. The recurring unit along the longitudinal axis between two successive Z lines is called the sarcomere (Fig. 1). Thus, the appearance of the striated muscle fiber is due to the arrangement of the proteins actin and myosin. Changes in the appearance of the bands takes place during contraction and relaxation. Such changes have been helpful in postulating various theories concerning the mechanism of muscular contraction at the molecular level.

A very brief description of skeletal muscle structure will follow. Excellent detailed descriptions of the structure have been presented by Perry (121) and Denny-Brown and collaborators (1).

Connective tissue sheathes surround each individual fiber and groups of fibers. Specifically, the epimysium connective tissue surrounds the whole muscle; the perimysium separates the muscle into fasiculi; and the endomysium surrounds individual fibers. Furthermore, the muscle fibers are enclosed by a thin transparent membrane called the sarcolemma. Muscle fibers contain nuclei, myofibrils, and sarcoplasm and are characteristically large, multinucleated cells that differ in size from muscle to muscle and within a single muscle. The nuclei are often located directly beneath the sarcolemma. The sarcoplasm, the undifferentiated protoplasm of the muscle fiber, occupies spaces between the myofibrils (1). Electron microscope studies have revealed four types of structures within the sarcoplasm--the sarcosomes, sarcoplasmic reticulum, small particles (glycogen), and lipid granules (Fig. 1). Sarcosomes (muscle mitochondria) are situated between myofibrils (Fig. 1). The sarcoplasmic reticulum consists of a continuous network of tubules, vesicles, and cisternae that lie around myofibrils (124, 125) (Fig. 1). Glycogen and lipid granules will be discussed in a subsequent section.

3. Energy Production

Another property of voluntary muscle that gives significance to its structural and functional properties is the manner in which muscle derives energy from chemical reactions within the cells. Studies by Meyerhof (106) demonstrated that during anaerobic contraction there is a direct relationship between work done and lactic

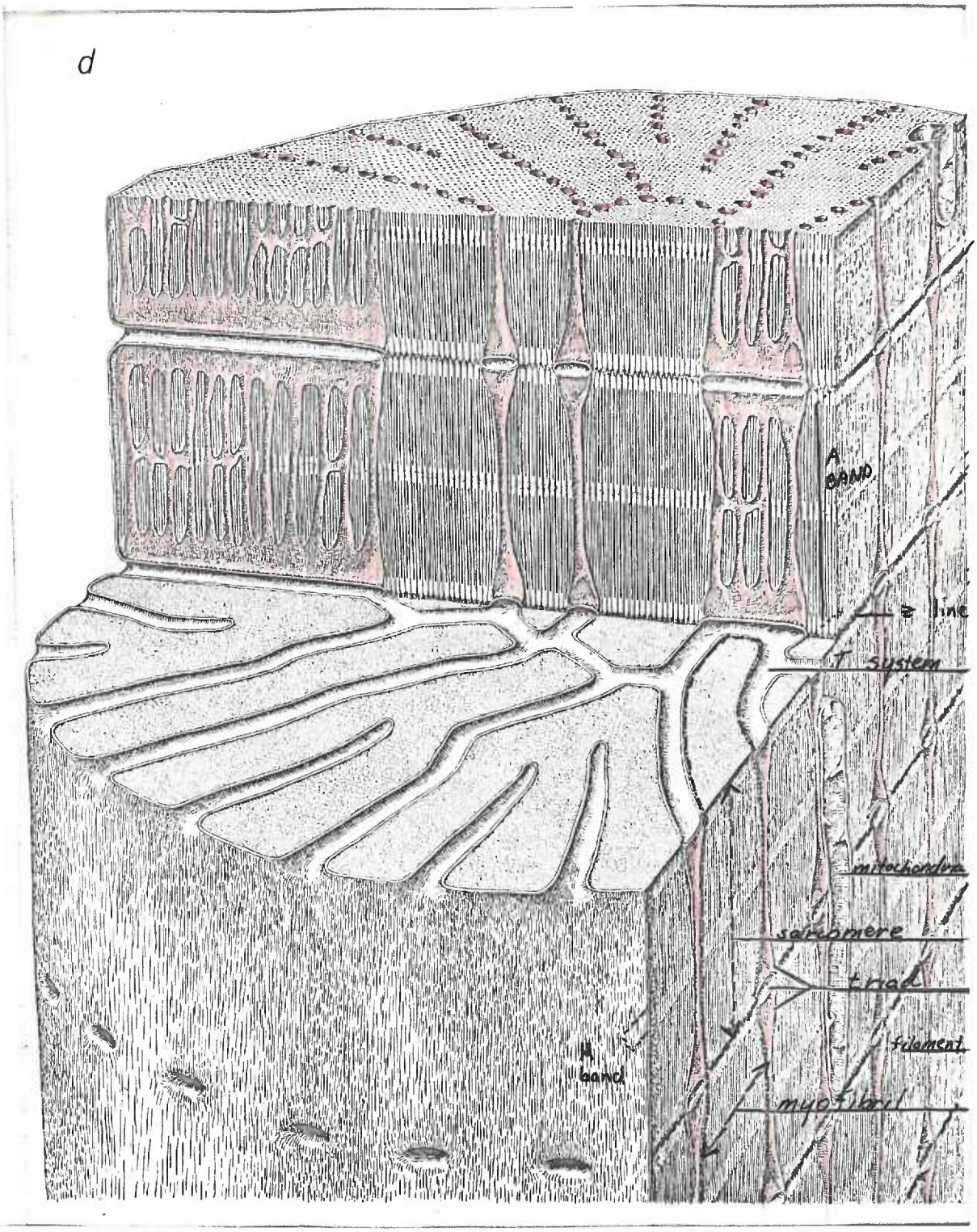


Figure 1. The Muscle Fiber(125)

acid formed. Fletcher and Hopkins (50) showed that lactic acid production accompanies fatigue and rigor in muscle. Parnas and Wagner demonstrated that muscle glycogen is a source of lactic acid (119). Under anaerobic conditions the breakdown of glycogen to lactic acid yields three molecules of adenosine triphosphate (ATP) from each glycosyl unit, while respiration and ATP formation (aerobic oxidation) produces thirty-nine molecules of ATP from each glycosyl unit. Oxidative phosphorylation, therefore, is a much more efficient process than glycolysis in the production of ATP. Another energy relation in muscle was demonstrated by Lundsgaard by the use of muscle poisoned with iodoacetate (102). Lundsgaard found that although iodoacetate inhibits glycolysis, the muscle continues to contract until the store of creatine phosphate is used up. Thus he suggested that the energy for muscle contraction comes ultimately from phosphocreatine. It is now known that phosphocreatine serves as a labile reservoir of high-energy phosphate groups which rephosphorylates ADP. The net effect of the energy producing systems is to maintain the ATP concentration of muscle at a high, steady-state concentration to serve as the direct energy source for contraction.

B. Historical Background--Red and White Muscle

1. Relation of myoglobin and muscle color

Reviews of Needham (111), Bullard (25), Björch (18) and Denny-Brown (37) give excellent accounts of the early investigations concerning red and white muscle. Among the early workers there were various ideas concerning the differences in color between red and

white muscle. Lorenzini (1678) observed marked differences in color between certain rabbit muscles (100). Boerhaave (1739) believed the red color is due to a greater blood supply in dark than in light muscles (22). Bichat (1803) suggested that muscle color is due to some substance which is closely associated with circulating blood and deposited in the muscle (17). Other workers (1810-1840) expressed similar ideas and suggested that there is a pigment connected with the muscular substance (18). Kühne (1865), using blood free extracts of rabbit muscle, found that the extract from pale muscle was colorless but that from dark muscle was red (88). This disproved previous ideas the red color is due to blood. Since Kühne prepared derivatives similar to those of hemoglobin, he concluded that the red color is due to this pigment. According to Needham (111), Kühne was the first to demonstrate that red muscle contains a larger amount of the pigment than white muscle. Lankester (1871) did not confirm Kühne's findings, and claimed that the color is due to a blood pigment diffusing into muscle (90). Meyer and Krause (105) suggested that the paleness of muscle color is a sign of degeneration due to domestication of animals. Since Kühne's work, Günther (1921) reported that the red pigment resembles hemoglobin but is not identical to it and suggested the term myoglobin (62). From such early investigations it was concluded that red or dark muscle contains a larger quantity of myoglobin than white or light muscle.

2. Relation of granular content to muscle color

The color of muscle is related not only to the content of myoglobin but also to the granular content of the sarcoplasm. Kölliker

(1851) described interstitial granules between myofibrils and suggested that they might be involved in metabolism (79). He recognized these granules to be both fat droplets and true interstitial granules (25). Krause (1864) observed that red muscle of rabbit contains more interstitial granules than does white muscle and he, therefore, related interstitial granules to muscle color (84). Grützner (1884) described two types of fibers in human muscle--one small and dark and the other larger and light (61). Most human muscles contain both types of fibers, and a greater proportion of the dark, smaller fibers gives the muscle a red appearance, partially due to the larger number of granules in the sarcoplasm (25, 111). Knoll (1889 and 1891) detected that the dark fibers are richer in interstitial granules and sarcoplasm than are the white fibers (77, 78). He suggested that the terms light and dark would be more appropriate than red and white, and pointed out that the color of the muscle fiber depends more upon the amount of myoglobin, fat, and other substances within the sarcoplasm than it does upon the amount of sarcoplasm (25, 111). Bullard (25) noted that the relative numbers of light, dark, and intermediate fibers is quite variable between various muscles of various species, but for a given muscle of a given species the percentage of each type is fairly constant.

3. Functional Differences Between Red and White Muscle

In addition to differences in color between red and white muscle, differences in function were noted by various scientists. Lankester (1871) suggested that red pigment is contained in muscle that undergoes prolonged and persistent action (90). In 1873 Ranvier correlated red

and white muscle of rabbits with slow and fast contractions (132). Grützner (61), using rabbit muscle, concluded that all smaller granulated fibers correspond to slow-contracting muscle and larger, clear fibers to more rapidly contracting muscle. Rollett (136) pointed out that although differences in function are often associated with differences in structure, such is not always the case. From diverse studies it was concluded that generally in lower animals the red, granular structure is confined to the most active muscles while only the least active are white. For example, in the domestic hen, which flies very little, the leg muscles are red while the breast muscle is white. About 1886 Adducco (2), Rollett (136), and Grützner (61) independently performed similar experiments on various animals and demonstrated that usually white muscle gives a higher twitch than does red muscle, but that red muscle gives a more prolonged tetanus than does white muscle. In 1908 Fischer (47) confirmed Grützner's observations using soleus (red) and gastrocnemius (white) muscles of the cat. Millikan (1939) found that both kinds of fibers occur in the same muscle, and agreed with previous work indicating that red muscle has a more prolonged twitch and more marked tetanus than does white muscle (107). More recent investigations have indicated a molecular structural difference between the myosins of red and white muscle based upon differences in myosin ATPase activity (142). From the investigations discussed, it has been concluded that red muscle is often tonic in its functional performance while white muscle is tetanic or phasic.

4. Chemical Differences Between Red and White Muscles

The demonstration of functional and structural differences

between red and white muscle led to the investigation of differences in chemical constituents. Needham (111) reported two isolated attempts to discover chemical differences between red and white muscle. Gleiss (1887) found that red muscle of guinea pig, rat, and cat became more acid than did the white muscle of these animals after activity or rigor mortis (57), and Knoll (1891) (78) mentioned the possibility of chemical differences between red and white muscle. Fletcher (49) reported that when mammalian muscle is allowed to stand at room temperature, white muscle produces lactic acid much more rapidly and to a greater extent as compared to red muscle. Furthermore, lactic acid production is important because Meyerhof and coworkers concluded that in frog muscle the breakdown of carbohydrate into lactic acid is a source of energy for muscle contraction. Quagliarello (127) claimed that white muscle of fowls and rabbits has a higher phosphate content than the red, but red muscle has more phosphatidyl phosphate. Riesser (133) detected a higher content of creatine and creatinine in white muscle as compared to red. Later work by Embden and colleagues (44) revealed that red muscle contains more cholesterol than white. Red muscle was also found to have a greater oxygen uptake.

5. Recent histochemical investigations in red and white muscle

With the development of new histochemical techniques, it became possible to characterize enzymatic differences between individual fibers. The enzymes involved in glycogen synthesis and breakdown such as phosphorylase, aldolase, and glycogen synthetase show different activities in different types of muscle fibers. Histochemical observations by Dubowitz and Pearse (41, 42) revealed that red fibers of

mammalian skeletal muscle stain less heavily for phosphorylase than do white fibers. In general, there is a reciprocal relationship between fiber staining for phosphorylase and succinic dehydrogenase--in other words, fibers which stain heavily for the glycolytic enzyme phosphorylase do not stain heavily for the oxidative enzyme succinic dehydrogenase. Bocek and Beatty (21) have pointed out that this reciprocal relationship cannot be demonstrated in the soleus of rat and monkey and is not universal. Another glycolytic enzyme, aldolase, was reported to be present at a higher activity level in white than in red fibers of pigeon (56). However, glycogen synthetase, an enzyme involved in synthesis of glycogen, has been shown by histochemical techniques to have a higher total activity in red muscle as compared to white muscle of the rat (21, 67) and rhesus monkey (21). Quantitative studies by Bocek and Beatty (21) have confirmed these histochemical observations. Beckett (13) has written an excellent review concerning the histochemistry of skeletal muscle which discusses the above points in greater detail.

Investigation of oxidative enzymes such as succinic dehydrogenase by histochemical and quantitative techniques has led to the general conclusion that fibers of skeletal muscle stain with varying intensity, but fibers of small diameter usually stain most intensely for the oxidative enzymes (12, 55, 114, 115, 118). However, enzyme variation does not always correlate with size since some workers have reported fibers of intermediate size and varying enzyme activity (12). Most of the evidence has indicated that oxidative enzyme activity is higher in red than in white fibers. Recent studies on muscle from rats and

rhesus monkeys indicate a direct correlation between histochemical observations and quantitative determinations of succinic dehydrogenase activity (12). More data has been obtained concerning the activities of succinic dehydrogenase and cytochrome oxidase than for the activities of other oxidative enzymes. This is partly due to the fact that one method for the localization of the sarcosomes is based upon the presence of succinic dehydrogenase in them and its histochemical detection (14, 109, 146). Generally, fibers which are reported to have a high succinic dehydrogenase activity also have high activities of other oxidative enzymes such as cytochrome oxidase (42, 115).

The major histochemical, physiological, and structural differences between red and white skeletal muscles of various species are summarized in Table I. Red muscle is rich in oxidative enzymes, myoglobin, lipid, and lipases, but poor in glycolytic enzymes while the opposites are true for white muscle.

Table 1. Some Properties of Red and White Muscle Fibers (42)

Property	Red	White
Diameter	smaller	larger
Color	red, dark, opaque	white, light, translucent
Myoglobin	high	low
Sarcoplasmic content	high	low
Mitochondria	numerous	sparse
Lipid	high	low
Lipase	high	low
Oxidative enzymes	high	low
Phosphorylase	low	high
Glycogen	low	high
Function	sustained activity	rapid activity
Speed of contraction	slow	fast

In view of the above differences in red and white muscle, it is evident that metabolic differences in these two types of voluntary skeletal muscle must also exist. It is the purpose of the experimental work in this thesis to elucidate further the differences in the metabolisms of red and white voluntary skeletal muscle. A discussion of the glycolytic and oxidative pathways in muscle is necessary in order to understand the previous and present investigations of glycogen metabolism.

C. The Embden-Meyerhof Pathway

The series of reactions by which glycogen or glucose is converted to lactic acid is termed glycogenolysis and glycolysis (Fig. 2). This pathway was first studied by Embden and Meyerhof in the period 1933-1936 (the name Embden-Meyerhof is often used). Investigations on the individual enzymes in the pathway were carried out by Warburg, the Coris, Needham, and others.

1. Glycogenolysis and Glycogenesis

Glycogen, a highly branched homopolysaccharide consisting of D-glucosyl units joined in α-1,4 and α-1,6 glucosidic linkages, is arranged in a tree-like structure within the tissues. When glycogen is isolated by mild quantitative procedures, a broad molecular weight spectrum is found with distinctly high and low molecular weight components (28, 117). The average molecular weight of mammalian skeletal muscle glycogen isolated by the mild procedure of cold water extraction is 5×10^6, while that of Ascaris has an average molecular weight of 180×10^6 (117). Much lower molecular weights have been

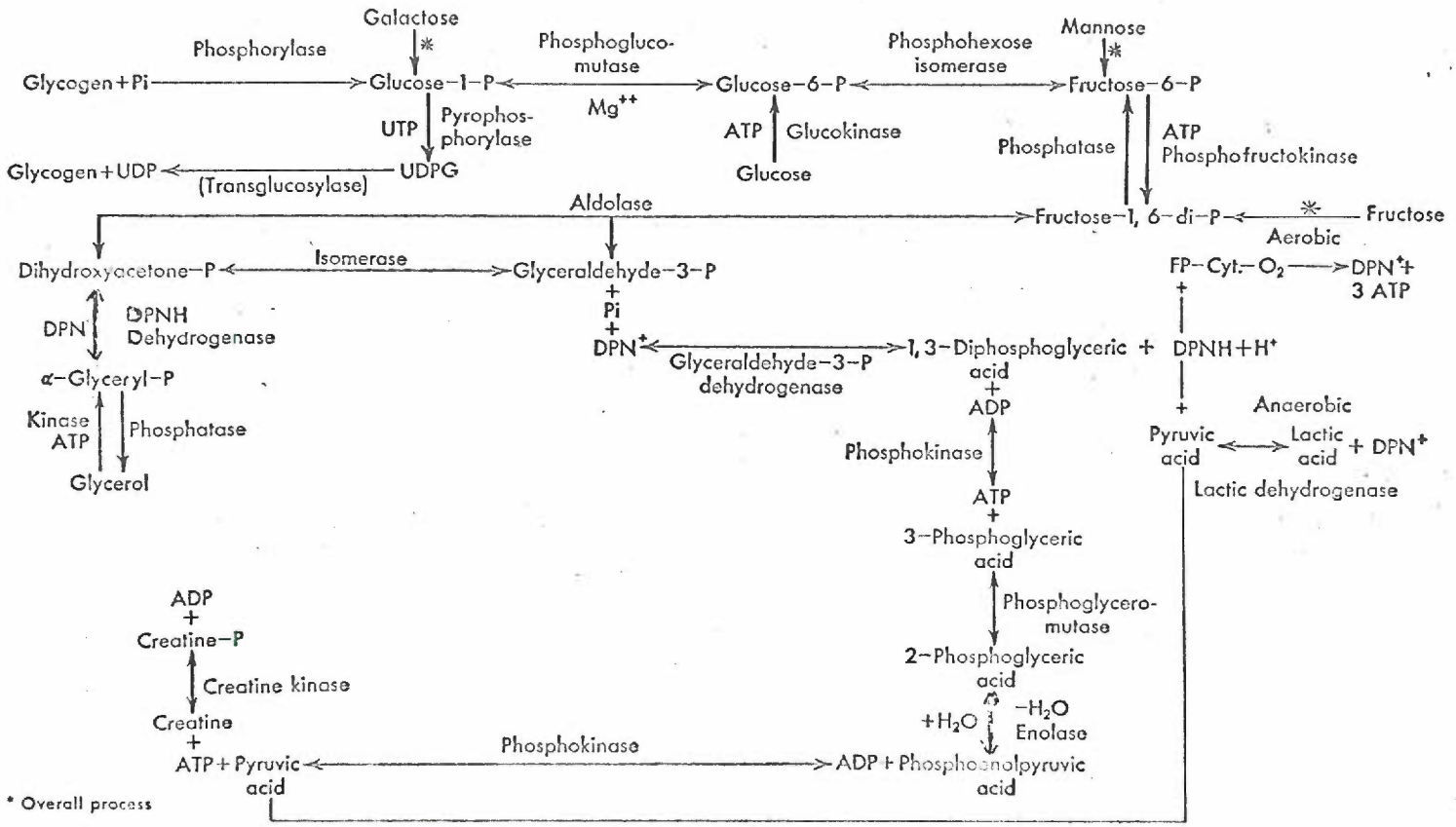


Figure 2. Glycolysis. Embden-Meyerhof Pathway

reported for glycogen isolated by cold trichloroacetic acid (TCA) or hot alkali (28, 117). Pertinent work on glycogenolysis has been done by Parnas, the Coris, Green, Fischer, Krebs, and many others (150). The phosphorylase enzyme initiates the breakdown of glycogen. This enzyme exists in two forms in muscle (59)---phosphorylase a (active form) and phosphorylase b (inactive form) that requires adenosine monophosphate (AMP) for activity (30). The phosphorylase phosphatase enzyme is responsible for the conversion of phosphorylase a to phosphorylase b as illustrated in the following equation:

$$\text{Phosphorylase a} \xrightarrow{\text{Phosphatase}} 2 \text{ phosphorylase b} + 4\ P_i$$

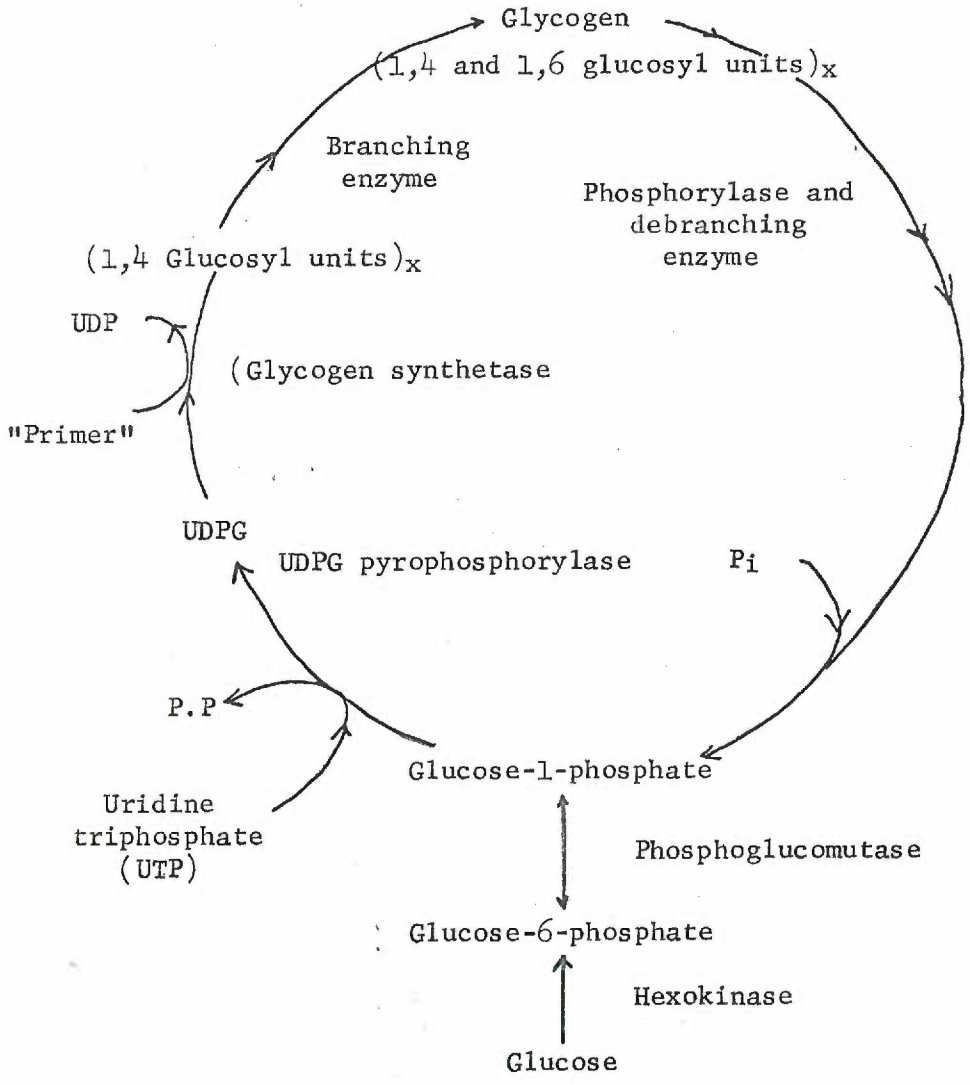
$$+ 4 \text{ ADP} \xleftarrow{\text{Kinase}} 4 \text{ ATP and } Mg^{++}$$

Keller and Cori (74) have shown that phosphorylase a has a molecular weight of 500,000 while phosphorylase b has a molecular weight of 250,000. According to Fischer and Krebs (46), phosphorylase a is a tetramer while phosphorylase b is a dimer. The conversion of phosphorylase b to a is mediated by the enzyme phosphorylase kinase (85). This reaction requires ATP and a divalent cation such as Mg^{++}. The phosphorylase protein molecule has interesting characteristics related to multiple binding sites on or within the molecule. This important aspect will be discussed in relation to control mechanisms.

Phosphorylase splits only the α-1,4 glucosidic bonds in the outer branches of the glycogen molecule. Hestrin (68) showed that muscle phosphorylase degrades glycogen and gives rise to a limit dextrin, which is attacked further by amylo-1,6 glucosidase(debranching enzyme) (31). More recently, it has been proposed that debranching

of the limit dextrin occurs through the action of amylo-1,6 glucosidase and a transglycosylase (66). The synergistic action of phosphorylase and debranching enzyme leads to the formation of glucose-1-phosphate (G-1-P) as indicated in Figure 3.

Figure 3. Glycogenolysis and Glycogenesis in Muscle



Recently it has been demonstrated that glycogenosis *in vivo* proceeds by a different route than by reversal of glycogenolysis. The accumulation of data by various investigators (150) was rendered meaningful when Leloir and Cardini (95) found an enzyme in extracts of rat liver homogenates that catalyzes the transfer of glucose from uridine diphosphate glucose (UDPG) on to a glucosyl-1,4 primer. Villar-Palasi and Larner (159) demonstrated the presence of UDPG-glycogen transglucosylase (glycogen synthetase) and UDPG-pyrophosphorylase in muscle. The former enzyme plus amylo-(1,4→1,6) transglucosidase (branching enzyme) (91), catalyzes the conversion of UDPG to glycogen, while the latter enzyme catalyzes the reaction of G-1-P and uridine triphosphate (UTP) to form UDPG (Fig. 3). The enzyme glycogen synthetase exists in two active forms that can be distinguished by their sensitivity to glucose-6-phosphate (51, 137). One form (glycogen synthetase I) acts independently of G-6-P while the other form (glycogen synthetase D) depends on G-6-P for its activity. The conversion of one form of the enzymes to the other involves phosphorylation-dephosphorylation:

a) Synthetase I + nATP $\xrightarrow{\text{kinase}}$ Synthetase D + nADP

b) Synthetase D + nH_2O $\xrightarrow{\text{phosphorylase}}$ Synthetase I + nP_i

Both glycogen synthetase and phosphorylase exist in more active and less active forms. The more active form of phosphorylase (a form) is phosphorylated while the opposite is true for glycogen synthetase. Larner and coworkers (93, 137) proposed that phosphorylase and glycogen synthetase are subject to simultaneous regulation through a common kinase or phosphatase and that stimulation of the phosphorylation

mechanism would result in an activation of one enzyme and an inhibition of the other. The significance of these findings will be discussed and integrated into the overall control mechanism of glycolysis.

2. The turnover rate of glycogen

The use of radioactive precursor substances in studies of glycogen metabolism poses a problem because the glycogen molecule is not metabolically homogeneous (147). Glycogen turnover is not a replacement of pre-existing molecules by newly formed molecules, but is first, a rapid process in which new glucosidic residues are added to the peripheral, nonreducing terminal ends of the pre-existing glycogen molecules and second, a slower process, involving the branching enzyme, in which initially peripheral glycosyl residues move into more centrally located portions of the glycogen molecule (147). Also, glycogen breakdown involves the removal of glucosyl units from outer branches of the glycogen tree, and the entire structure is not broken down. Consequently, glycogen does not have a true turnover rate. Muscle glycogen with a higher molecular weight and more branches incorporates glucose-C^{14} more rapidly than does glycogen with a lower molecular weight and fewer branches (28, 148). Therefore, the probability of a glucosyl residue being replaced within a given time interval depends upon its location within the glycogen molecule and the molecular weight of the glycogen (148). Such inhomogeneous labeling of glycogen complicates metabolic studies.

3. TCA soluble, residual, and total glycogen

Differences in solubility and extractability have often formed

the basis for the division of glycogen into two fractions. One glycogen fraction that is extracted by water or cold trichloroacetic acid (TCA) solution is called TCA extractable or soluble, free, lyo, or easily extractable glycogen. The other fraction, that requires alkaline or hot TCA digestion for extraction from tissues, is called residual, fixed, bound, or desmo glycogen. Some workers have assumed that the latter fraction is protein bound (150). There has been controversy as to whether there is a real distinction between TCA soluble and residual glycogen. Roe and coworkers (135) claim that the failure to remove all glycogen from tissues by extraction with cold TCA is due to inadequate homogenization. They used repeated homogenization with glass beads and found that essentially all glycogen was transferred from rat liver, heart, and gastrocnemius into the TCA medium. In regard to Roe's work, Kits Van Heijningen (76) states that the use of glass homogenizing beads probably breaks mitochondria and sarcosomes and allows destruction of large protein molecules and release of protein bound glycogen. The experiments of Russell and Bloom (138) indicate a physiological difference between the two fractions in rat tissues. They found that the TCA soluble glycogen of the gastrocnemius muscle of the rat accounts for about 56% of the total glycogen present, the other 44% being in the residual form. However, in the liver of the rat, these workers found about 85% of the total glycogen to be in the TCA soluble form. Other workers have reported similar findings in muscle. Although there is evidence for two glycogen fractions in muscle, Brown[1] has reported the presence of only one fraction in liver.

[1]D. H. Brown, Personal communication. Feb., 1966.

Various workers have attributed the presence of extractable and fixed glycogen in muscle to the choice of solvent, temperature, and other experimental conditions (64, 75), and it is questionable whether differences in solubility and extractability are substantial criteria for the division of glycogen into two fractions.

Not only has the existence of two separate fractions been questioned but also the metabolic significance of the two fractions. Many references are found in the literature concerning the effects of nutritional, hormonal, and other imposed variables upon the levels of the two glycogen fractions (150). The studies in this thesis have been performed to elucidate the metabolic significance of the two fractions. According to Stetten and Stetten (150), the free glycogen fraction exhibits the widest variation while the residual fraction remains more constant in quantity. Russell and Bloom (138) found that the TCA soluble glycogen is metabolically more active in gastrocnemius and heart muscle of the rat under conditions such as feeding, fasting, and excess or deficiency of hormones. However, Stetten et al. (149), who used labeled glucose, found the residual fraction of rat gastrocnemius to be more active metabolically. Kits Van Heijningen (76) states that both glycogen fractions of isolated rat diaphragm take up labeled glucose rapidly and, therefore, neither is more active metabolically than the other. Bocek et al. (19) indicate that the two glycogen fractions in red and white muscle of the rat differ metabolically in vivo and in vitro in that they incorporate glucose-C^{14} at different rates. In red muscle, the TCA soluble fraction incorporates more glucose-U-C^{14} while in white muscle the residual fraction

incorporates more. In the in vitro experiments the glycogen concentrations in both fractions of red muscle were similar, but in white muscle the residual glycogen concentration was higher than the TCA soluble glycogen concentration. Domonkos (39) utilized tonic (red) and tetanic (white) muscle of the rabbit and found a greater decrease in the glycogen concentration of tetanic muscle in vitro. Both muscle types showed similar decreases in free and fixed glycogen fractions during incubation--the free decreasing at a rate approximately twice that of the fixed.

Total glycogen levels in rat muscle obtained immediately after sacrificing the animal have been reported to be higher in white than in red muscle (6, 10, 19, 20, 114). However, the total glycogen concentration is lower in white muscle than in red after a two hour incubation (10, 19, 20, 39). Conversely, Bär and Blanchaer (6) in vitro obtained a net glycogen increase in both diaphragm (red) and external oblique (white) muscles of the unanesthetized rat incubated in media containing 200 mg % glucose. Their finding might be explained by the initial low glycogen levels which favored glycogenesis.

The uptake of labeled glucose from the medium in in vitro experiments was found to be greater for red muscle (1.90 mg/g/hr) of the rat than for white muscle (1.65 mg/g/hr)(20). Likewise, the percent of the glucose-C^{14} uptake appearing as glycogen was about 2.5 x greater for red (22.5%) than for white muscle (9.2%)(20). Although the glycogen concentration in rhesus sartorius (red muscle) is about 2x higher than that of the red area of the semimembranosus in the rat, the rhesus sartorius incorporates less of the label from the glucose-C^{14}

uptake into glycogen during 2 hours incubation (16%) (11).

4. Lactate and Pyruvate Production in Red and White Muscle

The production of lactate and pyruvate have often been used as a measure of glycolytic activity in muscle. As previously mentioned, it has long been observed that white muscle produces more lactic acid than does red muscle. Recent observation have confirmed this early finding. Lactate production is greater in white than in red muscle fibers of the rat (10); in tetanic versus tonic muscle of the rabbit (39); and in homogenates of white versus red muscle of the rabbit (114). These experiments have been interpreted to mean that glycolysis is more active in white as compared to red muscle (20). However, the 2.5x greater per cent incorporation of labeled glucose into glycogen of red muscle after a 2 hours incubation period seems to reflect a greater degree of glycogenesis in red than in white muscle. A larger amount of glycolysis might be expected in white than in red muscle since the former has been shown to be richer in the glycolytic enzyme phosphorylase (21). Similarly, red muscle might be expected to exhibit a higher degree of glycogenesis because it contains more glycogen synthetase enzyme (21).

5. Hexose Phosphates

Glucose or glycogen metabolism via the Embden-Meyerhof pathway involves a series of phosphorylated hexose compounds (Fig. 2). Various workers have determined the amounts of these phosphorylated glycolytic intermediates in the diaphragm (34, 112), unspecified skeletal muscle (34, 96, 153), heart (34, 112), liver (34, 45), and

brain (34, 101) of the rat. No data concerning hexose phosphate levels in red versus white muscle have been reported.

In most of the experiments on carbohydrate metabolism in muscle, it has been assumed that there is only one pool of G-6-P with the exception of Shaw and Stadie (139, 140) and of Landau and Sims (89, 141). The experiments of Shaw and Stadie indicated that in bicarbonate buffer the labeled glucose is a precursor for both glycogen and lactate while labeled G-6-P is only a precursor for lactate (rat diaphragm). These workers concluded that normal rat diaphragm contains two identical Embden-Meyerhof enzyme systems which differ in cytological location. Glycogen and lactate are formed via one pathway (of intracellular location) while only lactate is formed via a second pathway (possibly on the cell surface). These suggested pathways differ in their response to insulin. For example, the intracellular pathway is responsive to insulin while the pathway on the cell membrane is not. No further studies on the two separate Embden-Meyerhof pathways were done for the next seven years, until recent investigations by Sims and Landau (89, 141) partially confirmed the work of Shaw and Stadie. Sims and Landau reported their results in terms of the ratios of incorporation of C^{14} from glucose-1-C^{14} to that of G-6-P-1-C^{14} into lactate-C^{14}, $C^{14}O_2$, and glycogen-C^{14}. If there is only one G-6-P pool in the rat diaphragm, the label of G-1-C^{14} and G-6-P-1-C^{14} should be incorporated in the same proportion into each of the products. If the ratios of incorporation into the products are different, this would indicate more than one G-6-P pool. Critical to the use of these ratios is the

assumption that G-6-P is an obligatory intermediate in the metabolism of glucose (Fig. 2). Similar incorporation ratios were found for lactate and CO_2, but substantially higher ratios were found for glycogen. Thus, Landau and Sims concluded that lactate and CO_2 are primarily derived from one G-6-P pool while glycogen is obtained from another. Furthermore, they found that only the latter pool is insulin sensitive and proposed that the site of this pool might be the sarcotubular system (141). This suggestion is interesting since recent work has indicated a localization of insulin at the sarcolemma and possibly in its tubular extensions (43).

D. Oxidative Pathway

Pioneers in the study of the terminal pathway of oxidation in animal tissues include Thunberg, Batelli and Stern, Knoop, Wieland, Krebs, and Szent Györgyi. Krebs proposed a cyclic mechanism for the oxidation of carbohydrate in the 1930's. This major pathway functions in the oxidation of carbohydrate, lipid, and protein, and is known as the Tricarboxylic acid (TCA), Krebs, or Citric acid cycle. The substrate for the Krebs cycle in muscle and other tissues is acetic acid in an activated form, namely, acetyl coenzyme A. The latter can be formed from pyruvate, the aerobic product of glycolysis, and in this way a link between aerobic glycolysis and the Krebs cycle is maintained. Acetyl CoA is oxidized through a series of steps in the Krebs cycle ultimately to form CO_2 and H_2O.

Some of the reactions of the cycle have been known to exist in muscle since 1911, when Batelli and Stern found the rapid conversion

of citrate into succinate, malate, and fumarate in frog muscle. Moreover, they found that red muscle oxidizes succinate more rapidly than does white muscle. Other workers also found that oxidative processes are more intense in red than in white muscle. Thus, red muscle has been assumed to utilize the oxidative pathway to a greater degree for energy production than white muscle does. Recent experiments have confirmed this assumption, demonstrating that enzymes of the Krebs cycle are more active in red than in white muscle (42). Similarly, Ogata (114) demonstrated higher oxidative enzyme activity in homogenates of red muscle than in homogenates of white muscle from rabbit. Other investigators have reported that red muscle oxidized fatty acids by way of the TCA cycle to a greater extent than does white muscle (41). Beatty, Peterson, and Bocek (10) found a greater acetoacetic acid uptake by red as compared to white muscle fiber groups of rat, as well as greater incorporation of acetoacetic acid-C^{14} into $C^{14}O_2$ in red as compared to white muscle. They also noted a 32% greater O_2 consumption by red fibers. Domonkos and Latzkovits found that the O_2 consumption of tonic muscle (red) is 3x as great as that of tetanic muscle (white) of the rabbit (40). Furthermore, they demonstrated that red muscle utilizes more pyruvate and α-ketoglutarate than white does. More recent work has revealed that a higher per cent of metabolized glucose-C^{14} appears in the $C^{14}O_2$ of red muscle than in that in the white muscle of the rat (6, 19, 20). Thus, there are striking differences in the oxidative and glycolytic metabolism of red as compared to white voluntary skeletal muscle.

E. Insulin and Carbohydrate Metabolism in Muscle

Due to the extensive work concerning insulin and carbohydrate metabolism in muscle, only pertinent ideas will be discussed. Banting and Best (1922) prepared the first concentrated insulin extract from pancreas and noted that the level of blood sugar could be controlled with insulin (5). An increase in the rate of glucose oxidation and glycogen deposition in muscle of decapitated, eviscerated rats upon administration of insulin was demonstrated by Best and colleagues (16). Gemmill (54) showed that insulin causes an increase in glycogen synthesis and glucose uptake in the isolated rat diaphragm. Following the period when most of the major pathways for carbohydrate metabolism had been established, many workers began to investigate the effects of insulin upon carbohydrate matabolism. The work of the Coris (1945-1950) (126) and of Krahl (82,83) represented one of the first attempts to provide a general theory of the action of insulin on glucose utilization. Their proposal was that insulin exerts its effect by overcoming pituitary or adrenal inhibition of hexokinase activity. Other investigators could not confirm this work(144, 145). However, recently effects of insulin on liver and adipose hexokinase (24) have been demonstrated.

Levine and coworkers performed experiments infusing hexoses and pentoses into eviscerated-nephrectomized dogs (98, 99). These sugars were confined to the extracellular space in the absence of exogenous insulin, but upon administration of insulin the sugars entered the intracellular space. They concluded that insulin acts to facilitate the movement of glucose into the cell. This "permeability" or

"transport" hypothesis of insulin action has been confirmed by other investigators (108), and in muscle it is generally agreed that the primary effect of insulin is to stimulate the movement of glucose into the muscle cell. Morgan and coworkers (108) have shown in heart muscle that transport is the major rate-limiting step at low glucose concentrations in the absence of insulin, but that phosphorylation is rate-limiting when insulin and glucose are present. A different effect of insulin has been suggested by Randle and Smith (129). Since anoxia and cell poisons that inhibit oxidative phosphorylation and decrease high energy phosphate levels accelerate the transport of glucose into diaphragm, they suggest that when high energy phosphates such as ATP are abundant, the transport of glucose into the cell is restrained. Such a mechanism of sugar transport that acts to slow or inhibit the movement of glucose into the cell, has been called a "keeper-outase" apparatus (151). The function of insulin would then be to combine with the "keeper-outase" apparatus and allow glucose to enter the cell.

There is some experimental evidence that is inconsistent with the transport hypothesis. Chain et al. (15) have found that the insulin effect is largely on the synthesis of glycogen and oligosaccharides and does not significantly increase the production of lactic acid and CO_2. This group concludes that the enhanced movement of glucose into cells in the presence of insulin is an effect secondary to intracellular stimulation of glucose utilization, while the primary effect is an increased rate of G-6-P disposal by increased glycogen synthesis. However, it appears that their conclusion is questionable since insulin has been shown to increase the entry of metabolizable and nonmetaboliz-

able sugars into the muscle cell.

Glycogenesis and glycolysis and the rate of glucose phosphorylation are controlled by various factors to be discussed in the next section. Insulin stimulates a hexokinase enzyme in muscle (26, 27, 162), accelerates glycolysis at the level of the phosphofructokinase (PFK) reaction (112, 113), and stimulates reactions in the TCA cycle (65). Furthermore, insulin causes an increase in glycogen concentration that cannot be entirely explained by increased glucose utilization because glycogen retention is often beyond that expected from the increased glucose uptake alone (160). It has been demonstrated that insulin specifically increases glycogen synthetase I activity and thus increases glycogen synthesis (35, 160). Thus, the major effects of insulin on the carbohydrate metabolism of muscle include an increased glucose uptake or transport, increased glucose phosphorylation, and increased glycogen synthetase activity. Experimental evidence for the effects of insulin on all the various constituents of carbohydrate matabolism is too extensive to be thoroughly discussed in this thesis. The mechanism of insulin action has been the subject of numerous reviews (48, 81, 97, 128, 130, 131, 143, 163). The recent review by Randle et al. (131) and the monograph by Krahl (81) present excellent comprehensive discussions of the effects of insulin on carbohydrate metabolism.

F. Feedback and Control Mechanisms

The regulation of glycolysis in skeletal muscle is a subject of primary importance relative to any phase of muscle metabolism. Details of the control mechanisms are not yet known; however, in recent years

much pertinent work has been done, and a picture of the coordinated control of glycolysis is rapidly emerging. The idea of only one regulator of this multienzyme system is now obsolete. Rather it appears that there are multiple activators and inhibitors, the relative importance of which depends on the prevailing physiological conditions.

1. Product Inhibition of Glycogenesis and Glycogenolysis

Experiments by several investigators have indicated that glycogen exerts control over its own metabolism. Work by Danforth (35) on rat diaphragm and skeletal muscle and by Alpers (3) on HeLa cells suggests that glycogen controls its synthesis by product inhibition, via the glycogen synthetase enzyme. For example, the product, the glycogen macromolecule, enhances the enzymatic conversion of glycogen synthetase from the more active I form to the less active D form or vice versa. The mechanism by which glycogen affects interconversion of synthetase I $\longrightarrow$ D is unknown, but glycogen synthesis is favored when tissue glycogen is low, and conversely, inhibited when glycogen is high. In other words, glycogen synthetase I activity varies inversely with glycogen concentration. ATP and Ca^{++} (4) independently stimulate conversion of glycogen synthetase I form to D form, and therefore decrease glycogen synthesis.

The control of glycogen breakdown in muscle involves the enzyme phosphorylase, which also exists in two forms. Phosphorylase is known to contain several well defined regions which control the activity of the enzyme. These active sites that bind substrates or catalysts are the adenosine monophosphate (AMP) binding site that also competitively binds ATP, the phosphorylase b $\longrightarrow$ a conversion site that involves

seryl-phosphate, and the pyridoxal-5-phosphate cofactor site (46). The interactions of these sites are of importance in the regulation of enzyme activity. For instance, the phosphorylase molecule is inactive in the absence of AMP and P_i, but the presence of either produces activity. Interaction at the seryl-phosphate site, involved in the conversion of phosphorylase b to a in muscle, has been the subject of recent investigation by Fischer and Krebs (46), who found that partially phosphorylated intermediates (phospho-dephospho hybrids) exist. AMP treatment of these hybrids favors retention of the tetramer (phosphorylase a), while G-6-P abolishes this effect. It is not known whether such forms exist in vivo but it may be that the conversion of phosphorylase a to b does not always proceed to completion and such intermediates may exist. If this is the case, these hybrids might exert effective control on glycogen utilization due to their sensitivity to G-6-P, AMP, and G-1-P.

Since glycogen is in close physical association with both phosphorylase and glycogen synthetase (3), it is possible that glycogenolysis and glycogenesis are under a concerted control mechanism, and evidence for such a control mechanism has been accumulating. For instance, G-6-P has been found to enhance the activity of glycogen synthetase D (137) and suppress the activation of phosphorylase b (46). Similarly, ATP as well as Ca^{++} stimulate the conversion of glycogen synthetase I to D (4), and also stimulate phosphorylase kinase activity in the conversion of phosphorylase b to a (46). AMP increases the activation of phosphorylase b (108) and inhibits the activity of glycogen synthetase I. Thus there appears to be a feedback system in which a

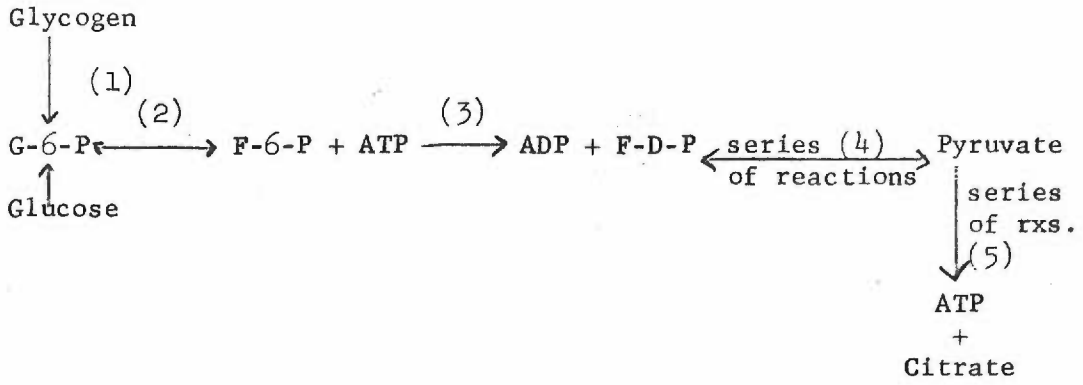
change in the level of glycogen can stimulate glycogen breakdown by increasing the more active form of phosphorylase and stimulate glycogen synthesis by increasing the more active form of glycogen synthetase. Evidence from studies involving insulin (35, 160) and epinephrine (86) provide further support for such a feedback control. Under the appropriate conditions insulin appears to convert glycogen synthetase D to glycogen synthetase I and thus stimulate glycogenesis (93).

2. Factors Influencing Overall Control of Glycolysis

The metabolism of glycogen is only one phase of glycolysis, and the control mechanism must be integrated with the other phases of glycolysis. The overall control of glycolysis has been studied by investigating two key glycolytic enzymes, hexokinase and phosphofructokinase (PFK). The current investigations indicate a coordinated stimulation of hexokinase and PFK by P_i (156, 157, 158). The pertinent experimental evidence will be discussed. Crane and Sols found that muscle hexokinase is inhibited noncompetitively by G-6-P (33). The reaction involved is as follows:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{G-6-P} + \text{ADP}$$

The inhibition of partially purified hexokinase from brain and ascites tumor cells by G-6-P is partially counteracted by P_i while the product inhibition of hexokinase by ADP is not relieved by P_i (157). Thus, hexokinase activity is governed by the product inhibition of G-6-P and ADP, both of which are competitive with ATP. The activity of the PFK enzyme is influenced by its end products and also by other metabolites. PFK catalyzes reaction 3 of the following series:



The PFK enzyme is unique in that it is inhibited by one of its substrates, ATP, and stimulated by the other, fructose-6-phosphate (F-6-P), (120, 157). At low concentrations the product FDP activates PFK, but at high concentration it inhibits the enzyme (120). Other activators of PFK include AMP, ADP, P_i, 3'5' cyclic AMP, and NH_4^+. Inhibition of PFK by citrate, P-enolpyruvate, and P-creatine, suggests a possible feedback inhibition by these intermediates as a means of regulating glycolysis (157). Work by Mansour has indicated the complexity of the PFK role in the control process. He found that PFK activation can occur by "kinetic activation" or "structural activation" (103). Studies on kinetic activation by substances such as cyclic 3'5' AMP indicate the presence of a multivalent system for enzyme regulation that involves the reversible dissociation of active enzyme to an inactive enzyme (103, 104). Another possible control mechanism under investigation is based upon the aggregation of the enzyme in a monomer-polymer system, with the monomer being the inactive form and the polymer the active form (103). A computer analysis of mammalian PFK has been made in order to study the control mechanism of this complex enzyme (53).

A concept of the PFK control mechanism is emerging in spite of the complexities in the process. Reports in the literature have suggested that the reaction controlled by PFK is the major rate-limiting step in the regulation of glycolysis. Uyeda and Racker (158) demonstrated in reconstructed systems that P_i stimulates mammalian glycolysis at three different sites and operates to 1). relieve the inhibition of hexokinase by G-6-P, 2). relieve the inhibition of PFK by ATP, and 3). stimulate the oxidation of glyceraldehyde-3-P (156, 158).

G. Purpose of Experimental Work

1. Preliminary Experiments

The problem under investigation concerns various aspects of glycogen metabolism in red versus white muscle of the rat and rhesus monkey. Previous work in this laboratory has demonstrated differences in the glycogen metabolism of red and white muscle of the rat (10, 19, 20). Under control conditions white muscle was found to have a higher glycogen level than red muscle (10, 19). Before I could begin the *in vitro* work on rhesus monkey muscle, it was necessary to determine glycogen levels in red and white muscle under resting conditions since no data were available in the literature. When muscle samples from rhesus monkeys were examined, it was found that the control glycogen levels of the soleus (red) and sartorius (predominantly red) were significantly higher than the brachioradialis (predominantly white) of the rhesus monkey. These observations did not agree with previous results in the rat or with reports in the literature for glycogen levels of red and white muscle of several mammals excluding primates.

Thus several series of glycogen determinations were performed under various conditions in an attempt to determine why the white muscle of the rhesus has a lower initial glycogen concentration than that of red muscle. An experiment was designed to investigate the lability of glycogen in red and white muscle. Since muscle samples were frozen in previous experiments, a comparison was made of total glycogen concentration in freshly biopsied muscle between frozen and nonfrozen tissue (II B 5 a). Also, TCA soluble and residual glycogen levels in samples of red and white muscle were investigated to determine if the lower glycogen concentration in white muscle might reflect a decrease in the glycogen in one of these fractions and in such determinations some thawing of the frozen sample was noted (II B 5 b). Therefore, the effect of freezing and thawing on the glycogen levels was determined. The results of these attempts (III A 1, 2) yielded interesting information; none of which explained the lower glycogen levels of white as compared to red muscle in the rhesus. However, the rhesus monkey is a highly excitable animal, and work in our laboratory indicates that stress causes a greater decrease in glycogen levels of white muscle than red. This fact might explain the low glycogen levels in white muscle of the rhesus monkey. Various techniques were employed to obtain muscle samples without stressing the monkey. Several different anesthetics (sernylan injected intramuscularly or pentothal given intravenously or by suppository) were used. To avoid the problem of catching the animal on the day of biopsy the monkey was placed in a chair for two weeks. However, we were never able to obtain a rhesus monkey with a higher glycogen level in the white than in the red muscle--that is, an

unstressed rhesus. This was surprising since previous data(21) indicated that differences in enzyme activities of phosphorylase a and b and glycogen synthetase I and D of red as compared to white muscle were similar in the rat and rhesus monkey. Since the rhesus monkey appeared to be unsuitable for the experiments on red as compared to white muscle, the experiments were necessarily contined using muscle from the rat. The rat diaphragm has often been used for the investigation in vitro of skeletal muscle metabolism. However, the diaphragm is unsuitable for the investigation of red and white muscle because it is a mixed muscle, an involuntary skeletal muscle, and in the rhesus the tissue is too thick to use without slicing. Thus, the sartorius was chosen as representative of predominantly red voluntary skeletal muscle of the rhesus.

2. Specific Problems Investigated

As previously indicated glycogen has often been divided into two fractions -- a TCA soluble and a residual fraction. Recent work indicates that the existence of a residual fraction of glycogen in liver and muscle is questionable (64,135). To determine if the TCA soluble and residual glycogen fractions in muscle are of physiological significance or merely artifacts of analytical techniques, variation in homogenization time, the temperature of homogenization, and reextraction of the residual glycogen fraction were investigated as well as the effect of insulin on glucose-C^{14} incorporation into these two fractions.

Insulin has been shown to stimulate glycogen synthesis in muscle by increasing the activity of glycogen synthetase I (35, 160). Our laboratory has demonstrated (histologically and by quantitative enzyme assay) the presence of a larger amount of total glycogen synthetase

in red as compared to white muscle of the rat and monkey (21). No work has been published comparing the effects of insulin on glycogen metabolism of red as compared to white muscle. Therefore, a series of Warburg experiments were planned to investigate the effect of insulin on the incorporation of glucose-C^{14} into the TCA soluble and residual glycogen of red as compared to white muscle. These experiments should also provide further information concerning whether the two glycogen fractions are physiological entities since the effect of the hormone insulin is being tested on an in vitro muscle system.

This laboratory has previously demonstrated that the incorporation of label from glucose-C^{14} into glycogen is ten fold higher in red as compared to white muscle of the rat (19). These results might be explained by assuming a higher turnover rate of glycogen in red muscle; however, no data on precursor pools were available. An initial step in detemining pool sizes was taken by determining levels of glucose-6-phosphate in the two types of muscle from the rat. Another problem related to the above experimental finding and the levels of glucose-6-phosphate is the possible existence of more than one glucose-6-phosphate pool in muscle. Most of the published work on carbohydrate metabolism of muscle assumes only one glucose-6-phosphate pool. Recently, the existence of insulin responsive and nonresponsive glucose-6-phosphate pools in rat diaphragm muscle has been reported (89, 141). The results of the investigation of the possible existence of more than one glucose-6-phosphate pool in red and white voluntary skeletal muscle of the rat and red muscle of the rhesus is reported in this thesis. The presence of one or more pools can be determined by the ratio of incorporation of the label of glucose-1-C^{14} to the label of glucose-6-

P-1-C^{14} into the products--glycogen-C^{14}, lactate-C^{14}, and $C^{14}O_2$. Similar ratios would indicate the existence of only one glucose-6-phosphate pool but differing ratios would indicate two or more pools. Furthermore, the experiments were designed to demonstrate the relative sensitivity of the glucose-6-P pools to insulin (responsive or non-responsive) as well as the degree of insulin response in red versus white muscle.

In summary, the main objective of the experimental work in this thesis is to further elucidate the metabolism of glycogen in red and white muscle by investigating:

1) TCA soluble and residual glycogen fractions as physiological entities in the red and white muscle of the rat.
2) The effects of insulin upon the two glycogen fractions in red and white muscle of the rat.
3) The levels of glucose-6-phosphate in red versus white muscle of the rat.
4) The possibility of more than one glucose-6-phosphate pool in red and white muscle of the rat and rhesus monkey.
5) Insulin responsive and nonresponsive glucose-6-phosphate pools in red and white muscle of the rat and rhesus monkey and any possible differences in the degree of response between red and white muscle.

II. Materials and Methods

A. Animals

Female Sprague Dawlwy rats (160-270 grams), male and female rhesus (Macaca mulatta) and pigtail macaque (Macaca nemestrina)monkeys (3.3-12.0 kg) were used in these studies. Rats were maintained on Purina rat chow and kept in a room at constant temperature of 24-26° C. Monkeys were maintained on a diet of Purina monkey chow plus cabbage (in lieu of vitamins) once a week).

B. Tissue Glycogen Determination

1. Methods of Sampling and Storage

Monkeys were fasted overnight, anesthetized with 1-(phenylcyclohexyl) piperidine hydrochloride (Sernylan, Parke, Davis, and Co.) (1-2 mg/kg intramuscularly) and brachioradialis, soleus, pectoralis, gluteus maximus, and sartorius muscle samples were removed at biopsy or obtained immediately after exsanguination. Usually, the muscle sample was cut into pieces, immediately frozen, and stored in liquid N_2 until time of analysis. However, in one series fresh biopsied muscle samples were analyzed immediately.

2. Determination of Total Glycogen

Muscle samples were placed in 15 ml centrifuge tubes, 0.5 ml of 30% KOH added, and the samples digested in boiling water for 20 minutes. Since fat can interfere with the glycogen analysis, all samples were fat extracted. Two ml of diethyl ether were added to the samples, the tubes vortexed, allowed to stand for ten minutes, centrifuged at 3000rpm for 10 minutes (0-5° C), International Refrigerated Centrifuge, Model PR-2), and the upper layer of ether and dissolved lipid carefully drawn off and discarded. This extraction

procedure was repeated twice. The tubes were capped with capillary ventilated stoppers and heated for 10-30 seconds in a boiling water bath to remove any remaining ether. The tubes were allowed to cool and then 0.7 ml of 95% EtOH was added to each sample. The tubes were restoppered, boiled for 30 seconds, and allowed to stand at room temperature for four hours. At the end of this period the glycogen was isolated by centrifuging for 15 minutes (3000 rpm, 0-5° C). The supernatant was discarded, and the tubes drained for 5 minutes on glass filter paper. The precipitate was washed with 1 ml of 95% EtOH, thoroughly mixed on the vortex, and centrifuged for an additional 10 minutes (3000 rpm, 0-5° C). The supernatant was again decanted, and the tubes drained. The glycogen was dissolved in 5 to 10 ml of distilled water, the samples were centrifuged for 5 minutes (3000 rpm 0-5° C) to remove any insoluble particles and then decanted into 16 x 100 mm test tubes. The aqueous solutions were either stored in the deep freeze (-23° C) or used immediately in the colorimetric procedure (II B 4) or in the glycogen-C^{14} determination (II E 7).

3. TCA Soluble and Residual Glycogen

The method for the determination of TCA soluble and residual glycogen was adapted from the method of Russell and Bloom (138). Muscle samples were placed in conical glass homogenizing tubes containing 1 ml of cold 10% TCA and homogenized with a tight fitting glass pestle for 1 minute (200-300 rpm, 0° C). The pestle was rinsed with 0.5 ml of cold 10% TCA, the tubes capped with parafilm and allowed to stand at room temperature for one hour with frequent

vortexing to assure an adequate extraction of TCA soluble glycogen. The tubes were centrifuged 10 minutes (3000 rpm, + 5°C), the supernatant decanted, 0.5 ml of cold 10% TCA added to the residue, and the precipitate resuspended by vortexing. The supernatant was decanted following recentrifugation (10 minutes at 3000 rpm, 0-5°C) and combined with the first supernatant collected. Four ml of 95% EtOH were added to the supernatant to precipitate the TCA soluble glycogen, the tubes vortexed, and then allowed to stand 1 hour at room temperature before being placed in refrigerator overnight. The recovery procedure was the same as that described in II B 2. One ml of 95% EtOH was added to wash the residue left in the homogenizing tubes, and the tubes were vortexed, recentrifuged for 5 minutes, decanted, and drained. Five-tenths ml of 30% KOH was added, the samples were digested for 20 minutes in boiling water, then cooled to room temperature, capped with parafilm, and placed in the freezer overnight (-23°C). The residual fraction was fat extracted, the glycogen precipitated with 1 ml of methyl alcohol and then dissolved in water.

4. Colorimetric Determination

The amount of glycogen present was determined by a modification of the anthrone method (134). Reagents used included 100 mg% anthrone (Nutritional Biochemicals Corporation) made up in 79% H_2SO_4 (830 ml concentrated H_2SO_4 added to 170 ml of H_2O, made up to volume when cold) and glucose standard (stock solution 150 mg glucose/100 ml in saturated benzoic acid). Two dilute working glucose standards containing 30 µg glucose/ml and 15 µg glucose/ml were run with each day's determination. Duplicate 1 ml blanks (distilled water), 30 µg and 15 µg glucose

standards, and aqueous glycogen samples were pipetted into 16 x 100 mm pyrex tubes which served as cuvettes. If the weight of muscle in a sample was high the samples were further diluted so that the glycogen concentration in the 1 ml aliquots would be in a range to yield optical densities of 0.100-0.400. Colorimeter tubes were placed in an ice bath. Five ml of anthrone reagent were pipetted into each tube, the tube vortexed, and replaced in the ice bath until all tubes were prepared. The tubes were stoppered with ventilated rubber stoppers, placed in a boiling water bath for 15 minutes, transferred to an ice bath for five minutes, and then placed in the dark for 15 minutes. Samples were read against the blank at 620 mμ on a Coleman, Jr. Spectrophotometer. A factor of 1.11 was used to convert from mg glucose/g of tissue to mg glycogen/g of tissue wet weight.

5. Variations in Procedures for Glycogen Determinations

a. Total Glycogen

In the preliminary glycogen determinations, frozen samples of the sartorius, brachioradialis, and soleus were removed from the liquid N_2 and weighed within 20 seconds (before thawing occurred). The tissues were prepared for analysis of total glycogen as previously described (II B 2). In a second series samples of sartorius, pectoralis, and gluteus maximus were obtained at biopsy. A piece of each muscle was immediately frozen (12-15 seconds after biopsy) in liquid N_2, while the other piece (fresh) was placed directly in a preweighed 15 ml centrifuge tube, weighed, 0.5 ml of 30% KOH added, and the remainder of the analysis was carried out as described previously (II B 2).

b. TCA Soluble and Residual Glycogen

In the initial experiments on TCA soluble and residual glycogen levels, frozen samples of sartorius and brachioradialis were removed from liquid N_2, quickly chopped into small pieces, and weighed in an homogenizing tube to which 1 ml of cold 10% TCA was then added. Since thawing of the frozen samples was noted during the chopping procedure, analyses were performed using frozen (0 time samples) and thawed (3 minute samples) to determine if the thawing affects the glycogen levels. A frozen piece of sartorius muscle was cut into two pieces while at liquid N_2 temperature. One piece was removed from the container, chopped into smaller pieces (during which it was allowed to thaw), transferred to a homogenizing tube, and weighed. One ml of 10% TCA was added exactly 3 minutes after the tissue had thawed. The remainder of the procedure was identical to that described in II B 3. The second piece of muscle was placed in a preweighed homogenizing tube containing 1 ml of frozen 10% TCA (liquid N_2), the tube reweighed, a long handled scalpel was introduced into the homogenizing tube held at liquid N_2 temperature, and the tissue pulverized (II B 2). Five tenths ml of cold 10% TCA was pipetted directly on top of the sample immediately upon removal from the liquid N_2 container, and the sample had no opportunity to thaw in the absence of TCA. Tissues were homogenized and glycogen isolated and determined as discussed in II B 3.

A series of glycogen determinations were made using various temperatures and times of homogenization. A special technique for grinding the muscle samples was used to assure a homogeneous sample of the sartorius muscle with minimum condensation of water. The

frozen muscle sample was placed in a cold box lined with tanks containing dry ice (8). The temperature in the box was maintained at -55 to -60°C and the sample ground to a fine homogeneous powder in a stainless steel container by use of a power driven pestel. An aliquot of powdered muscle was transferred to the homogenizing tubes and 0.5 ml of 10% TCA added to the powdered sample prior to homogenization. Triplicate samples were homogenized at maximum speed for a period of 1 minute at 0°C, 2 minutes at 0°C, or 1 minute at 35°C. The determination of TCA soluble and residual glycogen was carried out by the usual method (II B 3).

Two additional variations in technique were employed to investigate the effect of reextraction on the amount of TCA soluble glycogen present. Frozen monkey sartorius muscle samples were prepared as were the 0 time samples. The identical procedure for the determination of the TCA soluble and residual glycogen fractions was used (II B 3) except that at the step of the alcohol wash, 0.5 ml of cold 10% TCA was added to the residue followed by a one minute rehomogenization of the residue at maximum speed (drastic procedure) or by a milder reextraction procedure in which the residue was resuspended in 10% TCA and vortexed for 30 seconds. In both the drastic and mild reextraction procedures, the TCA soluble glycogen and the residual glycogen were determined as previously indicated (II B 3).

C. Insulin and Glycogen Fractions

A series of Warburg experiments were designed to investigate the effect of insulin on the incorporation of glucose-C^{14} into the TCA soluble and residual glycogen fractions of red as compared to white

muscle of the rat.

1. Flasks, Incubation Media, and Radioactivity

One ml of Krebs bicarbonate buffered medium containing 0.52 mM $CaCl_2$ and 0.64 mM $MgSO_4$ plus 150 mg% glucose(155) was pipetted into 8 ml Warburg flasks and a small amount of media was tipped into the side arm. Twenty-five µl (50 munits) of insulin[1] (beef, 23.8 units/mg, 0.0003% glucagon by wt) or 25µl of distilled water was added to the side arms of flasks. Fifty µl (3.5 µc/ml) of D-glucose-U-C^{14} (SA[2] 13.9 mc/mM, New England Nuclear Corporation) was added to side arms of all flasks except equilibration flasks. For each experiment two media blanks containing medium and glucose-U-C^{14} were prepared.

2. Tissue Sampling

Rats were starved 18-20 hours prior to sampling and injected intraperitoneally with sodium pentobarbital (pentosol, H. C. Burns Co.) (2.5 mg/100 g body wt). A period of 10 minutes after injection was sufficient time to insure muscle relaxation. Rats were then decapitated and the skin was stripped back exposing the muscles of the medial surface of the leg. Since the animals were exsanguinated, it was easy to identify the white and red areas of the semimembranosus(60). The muscle was gently teased into groups of fibers about 30-40 mm in length and 1 mm in width. These fiber groups were placed in Krebs bicarbonate media (room temperature) without substrate and aerated with 95% O_2-5% CO_2 during the remainder of the dissection. Red and white muscle fiber groups were obtained from both hind limbs.

[1]Insulin was a gift from Dr. Mary A. Root.

[2]The activity or decay rate of a radio isotope per unit of mass of the sample.

3. Experimental Procedure

An experimental run consisted of 12 flasks, 6 containing red muscle and 6 white muscle. The flasks were gassed for 5 minutes (95% O_2 - 5% CO_2) in the Warburg bath and then equilibrated for 15 minutes. After equilibration duplicate flasks containing red and white muscle were removed as controls for TCA soluble and residual glycogen content. The C^{14} activity and the insulin were tipped into the main chamber of the remaining flasks, and these flasks were incubated for 10 minutes. At the end of the incubation period 0.1 ml of 6N H_2SO_4 was added directly into the main chamber to terminate the reaction.

4. Glycogen Determinations

The muscle was removed from the Warburg flasks, washed in distilled water, and frozen for later analysis. The subsequent steps in the preparation of the TCA soluble and residual glycogen fractions are the same as those described in section II B 3.

For the glycogen-C^{14} assay triplicate 0.2 ml aliquots of the aqueous samples of the TCA soluble and residual glycogen were plated and counted as will be described in II E 4, 7.

D. Determination of Glucose-6-Phosphate Levels in Rat Muscle

1. Tissue Sampling

Rats were fasted overnight, injected intraperitoneally with pentosol (4.0 mg/100 g body wt) and dissection was performed as previously described (II C 2) except the animals were not decapitated. The red and white areas of the semimembranosus and caudofemoralis were removed and frozen immediately in liquid N_2.

2. Preparation of Homogenizing Tubes and Tissue Samples

One ml of 0.5 N perchloric acid (PCA) was added to glass homogenizing tubes (Kontes B, conical tip). The tubes were capped with parafilm and placed in a stainless steel insulated container chilled with liquid N_2. The tubes were removed from the container, capped, wiped free of moisture, quickly weighed, recapped, and replaced in liquid N_2. All instruments used to powder the frozen tissue were precooled to liquid N_2 temperature. Powdering of the frozen sample was done in the insulated container (25 x 24 cm) at liquid N_2 temperature according to the following procedure:

1). A frozen muscle sample was placed in the mortar and chopped into several smaller pieces.

2). The muscle pieces were ground using a ceramic pestle until a homogeneous, fine powder was obtained.

3). About 75-150 mg of the powdered muscle was transferred with a calibrated spoon to a preweighed, shelled homogenizing tube.

4). The tubes were removed from the liquid N_2, corked, wiped free of moisture, quickly weighed, and replaced in liquid N_2. Tissues were homogenized as soon as all samples had been weighed.

Later, the procedure for powdering of the muscle sample was simplified. Similar results were obtained so this procedure was continued. Homogenizing tubes contained 1.0 ml of 0.5 N PCA frozen in the bottom rather than formed in a shell. A piece of frozen tissue was placed in the tube on top of the frozen PCA. The tubes were quickly weighed as

before, a number 10 blade fitted on a long handled scalpel was inserted in the homogenizing tube, and the tissue was finely divided by a chopping motion.

3. Homogenization, Extraction, and Neutralization

As soon as tubes were removed from the liquid N_2, 0.5 ml of 0.5 N PCA was placed on top of the frozen powder, and the tissue was homogenized for 1 minute (200-300 rpm). The tubes were held in a beaker of ice water during the homogenization. The pestle was rinsed down with 0.5 ml of 0.5 N PCA, and the homogenate was allowed to stand at room temperature for 15 minutes with frequent mixing to assure adequate extraction. Seven-tenths ml of a neutralizing mixture (1.3 M K_2CO_3 and 2.3 M $KHCO_3$) was added (73). The potassium perchlorate was precipitated by placing homogenizing tubes in ice (0° C) for 10 minutes, and then removed by a 10 minute centrifugation (3000 rpm 0° C). The neutralized clear supernatant fluid was used for the assay.

4. Assay Procedure

The assay method is specific for the determination of glucose-6-P (G-6-P) by the reduction of triphosphopyridine nucleotide (TPN) by glucose-6-P dehydrogenase (G-6-PDH) (80). The overall reaction is:

$$\text{Glucose-6-P} + \text{TPN}^{+} \xleftrightarrow[\text{G-6-PDH}]{} \text{6-phosphogluconolactone} + \text{TPNH} + \text{H}^{+}$$

The amount of TPNH formed can be measured by following the increase in optical density at 340 mμ in a Beckman DU spectrophotometer (70, 110, 112).

Before beginning the series of analysis for G-6-P levels, the

optics of the Beckman DU spectrophotometer were tested by using TPNH standards (Sigma Chemical Company). An appropriate amount of the Tris buffer pH 8.0 was added to a vial containing a preweighed amount of TPNH and the solution read at 340 mμ. Since a 97.3 % recovery was found, the optics of the system were assumed to be in good order. Also, G-6-P standards (disodium salt with 3 H_2O, assay 98%, Sigma Chemical Company) of 0.033, 0.05, and 0.1 μmoles G-6-P per ml assay mixture were run to test the assay system and a linear relationship was obtained. Since the extinction coefficient for 1 mM G-6-P is 6.22 (1 cm light path, 340 mμ), 0.1 μmoles of G-6-P per ml of assay media gives an optical density reading of 0.622 if the system is functioning properly.

a. Assay Mixture

The assay mixture was essentially a modification of that of Nahara and Ozand (110) and Newsholme and Randle (112). The assay media contained 0.12 M Tris buffer pH 8.0, 0.15 M disodium ethylene-dianinetetraacetate (EDTA), 36 mM TPN (Calbiochemicals monosodium salt with 86% assay), 4.5 mM glutathione (GSH), 4.5 mM $HgCl_2$, and 0.7 units of G-6-PDH (Calbiochemicals, yeast in 3.3 M ammonium sulfate, SA 140 units/mg). A one to one mixture of 9 mM GSH and 9 mM $HgCl_2$, made fresh daily, was used to selectively inhibit the glutathione reductase (110). All reagents were prepared in glass distilled water and stored at + 4° C. According to Hohorst (70), all solutions remain stable for several weeks under these conditions.

The amounts of the reagents used in the assay mixture (at room

temperature, 24-26° C) are indicated. Beckman silica cuvettes (1 cm light path, vol. 3.8 ml) were used, the total volume in each cuvette being 3.01 ml.

Reagents	Blank	Standard	Sample
0.12 M Tris buffer, pH 8.0	2.6 ml	2.5 ml	2.4 ml
0.15 M EDTA	0.2	0.2	0.2
36 mM TPN	0.1	0.1	0.1
4.5 mM GSH - $HgCl_2$ mixture	0.1	0.1	0.1
3 mM G-6-P	---	0.1	---
G-6-PDH	0.01	0.01	0.01
PCA muscle extract	---	---	0.2

b. Glucose-6-Phosphate Determination

An experimental run consisted of a blank, a G-6-P standard, and two unknown samples. The reaction was initiated by the addition of 0.2 ml of PCA muscle extract to the unknown samples or by 0.1 ml of 3 mM G-6-P to the standard. The samples were quickly mixed with small plastic stirring rod and read against the blank at 2 minute intervals. The assay was continued until a constant optical density reading was reached (10-30 minutes). The amount of G-6-P present was expressed in μmoles/g of tissue wet weight. A series of dry weight determinations on the muscle powder was done to determine if, during the original pulverizing procedure, moisture was condensing on the frozen muscle powder and thereby invalidating the tissue weight. Tissues were dried to a constant weight for 24 hours at 95° C and the percent dry weight was calculated. The average dry weight for the muscle powder is 23.4% ± 0.6% SE as compared with that for fresh muscle of 23.7% (19) which indicates that the pulverized muscle sample was not being diluted by

condensation of moisture.

E. Determination of the Number of Glucose-6-Phosphate Pools and Their Response to Insulin

1. Flasks, Incubation Media, and Radioactivity

Warburg flasks (double side arms, 22 ml volume) were used. Krebs bicarbonate buffered medium was prepared as described in II C 1. The experimental design was similar to that of Landau and Sims (89, 141). Krebs bicarbonate buffered media containing 2 mM G-6-P and 10 mM glucose was used; the flasks contained 2 μc of either D-glucose-6-phosphate-1-C^{14} (G-6-P-C^{14}) disodium salt (SA 2.98 mc/mM, Nuclear Chicago Corporation) or D-glucose-1-C^{14} (G-1-C^{14}) (SA 3.34 mc/mM, New England Nuclear Corporation). To compensate for the additions of G-6-P and glucose with the radioactive substrates, two medias were prepared containing nonlabeled G-6-P or glucose to bring the final concentrations of each substrate to the desired molarity.

Two ml of the appropriate media were pipetted into each flask. A small amount of the media was tipped into one side arm, and either 0.05 ml (0.1 units/ml) of insulin (Lilly Research Laboratories, HGF free) or 0.05 ml of doubly distilled water were added to the media in this side arm. Two μc of the appropriate labeled compound (0.05 ml G-6-P-1-C^{14} or 0.03 ml of G-1-C^{14} and 0.02 ml of doubly distilled water) were also pipetted into the side arm. This side arm was closed with a glass venting plug while the other side arm was closed with a rubber vial closure. The contents and arrangements of the flasks are as illustrated:

	Blanks		Red Fibers				White Fibers			
Flask	A	B	1	2	3	4	5	6	7	8
Krebs bicarbonate medium, pH 7.4	+	+	+	+	+	+	+	+	+	+
G-6-P-1-C^{14}	+	-	+	-	+	-	+	-	+	-
G-1-C^{14}	-	+	-	+	-	+	-	+	-	+
Insulin (100 mU/1ml)	-	-	-	-	+	+	-	-	+	+

2. Tissue Sampling

Rats were fasted overnight, anesthetized, and red and white muscle samples were removed and prepared as described previously (II C 2). Monkeys were also fasted overnight, anesthetized, and the sartorius muscle was removed at biopsy or immediately after exsanguination as described (II B 1). The sartorius muscle was pinned to a paraffin block, excess fat and connective tissue trimmed away, and muscle fiber groups (5-8 cm. in length) dissected by means of a pointed scalpel and placed in aerated Krebs bicarbonate buffer.

Separated muscle fiber groups from the rat or monkey were blotted on filter paper, placed in the flasks, and the flasks were reweighed and gassed (95% O_2-5% CO_2) for 15 seconds. The weight of the samples ranged from about 100 to 160 mg per flask. Dry weight determinations (II D 4) were performed on all series. Per cent dry weights for mixed muscle fibers of the rat are 16.5% ± 0.2% SE[1], N = 12 and for sartorius muscle fibers of the rhesus monkey are 18.4 ± 0.2% SE, N = 6.

3. Experimental Procedure

The initial experimental procedure was similar to that described

[1]Standard error of the mean.

previously (II C 3) except all flasks were incubated for two hours. At the end of the two hour incubation period, 0.3 ml of 9.5 N NaOH was added with a syringe and needle via the rubber vial closure into the side arm. One tenth ml 18 N H_2SO_4 was then added through the vial closure into the main chamber to terminate the reaction. Flasks were incubated an additional forty minutes to insure maximal absorption of $C^{14}O_2$ by the alkali.

In many of the experiments additional flasks with mixed muscle fibers were run to determine if leaching of glycolytic enzymes into the media had occurred. The procedure followed was essentially that of Landau and Sims (141). Flasks containing mixed muscle fibers were attached to manometers, gassed for 5 minutes, and incubated for 1 hour. The fibers were then removed from the flasks, G-6-P-1-C^{14} activity was tipped in, and the flasks incubated for 2 hours as described for red and white fibers.

4. Radio Assay

Radioactivity was measured using a Tracerlab Ominguard low background counter with a 2 inch window on 0.2 ml aliquots of samples plated in triplicate on planchets (1 1/4" diameter x 3/32" deep), background 0.81 cpm ± .02 SE, N = 153. Small disks of lens paper (1 1/8" diameter) were placed on the planchet. One-tenth ml of alkaline glue (pH 9-10), containing 5 g of glucose dissolved in 95 ml of 20% ethyl alcohol and 5 ml of 1% aerosol, was pipetted onto the lens paper to promote an even adherence of the lens paper to the planchet. The plates were dried for 30 minutes in an 85° C oven. All samples were

counted in triplicate for at least 3200 counts (usually 10,000 counts) and dilutions were such that no activities less than 40 cpm above background were counted.

The per cent efficiency for dry plating was determined by plating samples of Na_2CO_3 standard calibrated against the National Bureau of Standards C^{14} standard (Tracerlab) and glucose-C^{14} supplied by New England Nuclear. This method had an efficiency of 22.4%. The counting efficiency of the different filtrates was 16-20%. All results were corrected to an efficiency of 22.4%.

5. $C^{14}O_2$ Assay

The NaOH was removed from the side arm with rinsing and diluted to 3, 4, or 5 ml. Two tenths ml aliquots were plated for counting. $C^{14}O_2$ blanks were run to determine the amount of $C^{14}O_2$ activity released in the absence of metabolizing muscle. The activity in the blanks (G-1-C^{14}, 31 cpm ± 2 SE, N = 14 and G-6-P-1-C^{14}, 44 cpm ± 9 SE, N = 10) was subtracted from the $C^{14}O_2$ produced in the flasks containing tissue. The effect of different alkali concentrations on the $C^{14}O_2$ counting efficienty was determined by adding NaOH plus a known amount of $Na_2C^{14}O_3$ (SA 5.0 μc/mM, New England Nuclear Corp.) and diluting to 3, 4, and 5 ml. The results were compared to values obtained with a known amount of $Na_2C^{14}O_3$ diluted with 0.1 N NaOH. The relative efficiencies were 3 ml, 95.0% (N = 9), 4 ml, 92.6% (N = 14), 5 ml, 93.7% (N = 9). To test the recoveries of $C^{14}O_2$ under the conditions of the Warburg experiment, 0.25 μc of $Na_2C^{14}O_3$ was added to 2 ml of Krebs bicarbonate buffer pH 7.4, containing 2 mM G-6-P and 10 mM glucose. The procedure was similar to that described in II C 3.

Flasks were acidified and shaken with NaOH in the side arm for 40 minutes, 1 hour, and 2 hours. The per cent recoveries ranged from 58-66 with no significant increase after 40 minutes. Therefore, samples were shaken for a 40 minute period after acidification.

6. Lactate-C^{14} Assay

The lactate-C^{14} assay was adapted from a modification of the method of Barker and Summerson for the quantitative determination of lactates (7). Duplicate 0.2 ml aliquots of media were pipetted into tubes containing 1.5 ml water and 0.5 ml saturated (about 40%) $CuSO_4$. One gram (± 10 mg) of $Ca(OH)_2$ (powdered, Baker and Adamson) was added to each tube. Tubes were mixed on the vortex for 30 seconds, capped with parafilm, allowed to stand at room temperature for 30 minutes, and then centrifuged for 15 minutes (3000 rpm, 18-20° C). The supernatant was decanted and recentrifuged for 5 minutes. Triplicate samples of 0.2 ml of this supernatant were plated as described in II E 4. Lactate blanks were run on media containing C^{14} labeled substrate to determine the radioactivity carried over in the precipitation procedure. The C^{14} activity found in the blanks (G-1-C^{14}, 119 cpm ± 6 SE, N = 13 and G-6-P-1-C^{14}, 114 cpm ± 6 SE, N = 11) was subtracted from the lactate-C^{14} values in flasks containing muscle. Recoveries for the lactate-1-C^{14} standards (DL Lactic acid-1-C^{14}, Na salt, SA 1.6 mc/mM, International Chemical and Nuclear Corporation) were 76-81% (N = 22).

7. Glycogen-C^{14} Assay

Muscle samples were removed from flasks, rinsed twice in distilled water, drained on filter paper, placed in 15 ml centrifuge tubes, and 0.5 ml of 30% KOH (made fresh daily) was added. The procedure for the

precipitation of the glycogen is as described in section II B 2. Glycogen-C^{14} activity was determined on 0.2 ml aliquots of the aqueous glycogen sample. Triplicate samples were plated for counting on the Tracerlab according to the procedure given in section II E 4.

8. Determination of the Hydrolysis of Glucose-6-Phosphate to Glucose

Since hydrolysis of the G-6-P-1-C^{14} to G-1-C^{14} would alter the interpretation of the results of the ratios of incorporation of the C^{14} label from the glucose and G-6-P substrates into the various products, it was necessary to determine if hydrolysis occurred during incubation. Gel filtration in a column (33 cm x 2 cm) packed with a crossed-linked dextran (Sephadex G-10, Pharmacia, Uppsala, Sweden) was employed to separate G-6-P-1-C^{14} from G-1-C^{14}. The Sephadex acts as a molecular sieve first allowing the larger molecules to pass through the column while the smaller molecules are retained. Nine-tenths ml fractions were collected and 0.05 ml of each fraction was plated on filter paper discs (Whatman No. 42), placed on the bottom of a glass counting vial, and dried in an 85° C oven for 20 minutes. Five ml of scintillation fluid (0.4% 2,5 diphenyloxazole, 0.01% 1,4 bis-2(5-phenyl-oxazolyl)-benzene in toluene) were added and the samples counted for 10 minutes each on a Tri-Carb Liquid Scintillation counting system (Packard). A C^{14} standard and triplicate blanks containing a filter paper disc and scintillation fluid were run each time (900 volts, maximum gain settings, and channel settings from 100-800). The efficiency of the scintillation counter under these conditions is 62.2%.

To standardize the column solutions of G-6-P-1-C^{14} (1 μc/ml)

and G-1-C^{14} (1 μc/ml) separately and combined in Krebs bicarbonate buffer (composition, II C 1) were passed through the gel to determine if adequate separation occurred. Two distinct peaks (about 10,000 cpm above background) were obtained for the labeled compounds, the first peak represented G-6-P-1-C^{14}. The recovery of the labeled G-6-P by this method was 90%. Samples of medium (after 2 hours incubation) were centrifuged, passed through the column, plated, and counted. No H_2SO_4 was added to terminate the reaction since such an addition might promote hydrolysis of G-6-P-1-C^{14} to G-1-C^{14}.

F. Statistical Analysis

Statistical analyses were performed on the results using Student's "t test" at the 0.95 confidence level for the comparison of paired observations or means. Differences are not considered significant unless the probability value (P) is less than 0.05.

III. Results

A. Glycogen Determinations

1. Total Glycogen Levels in Red and White Muscle of the Rhesus Monkey

Since no values for the concentration of glycogen in red and white muscle of the rhesus monkey were available in the literature, total glycogen concentrations of the soleus (red), sartorius (predominantly red), and the brachioradialis (white) muscles were determined (Table 2). Glycogen concentrations were lower in brachioradialis (white) muscle than in soleus and sartorius (red muscles) and, similarly, there was no significant pattern observed between glycogen levels of other red and white muscles in another group of experiments (Table 3). These results were not in agreement with reports in the literature for other species such as the rat and the rabbit in which glycogen level of predominantly white muscle is always higher than that of red under resting conditions.

If glycogen of white muscle is more labile than that of red, the lower levels in white muscle of the rhesus might be explained by a more rapid decrease in the glycogen content in the interval between biopsy and freezing in liquid N_2 (about 45 seconds). To test this possibility, biopsies of the gluteus maximus (white), pectoralis major (predominantly white), and sartorius (predominantly red) muscles were obtained. One portion of each muscle was frozen within 12-15 seconds after biopsy and then processed as described in II B 5, while the other portion was maintained in the fresh state (unfrozen for 2-5 minutes) before alkaline digestion (II B 5). The results of these experiments (Table 3) indicate that there was no difference between

Table 2. Comparison of Total Glycogen Levels in Red and White Muscle of the Rhesus Monkey

Muscle[1]	N[2]	Total Glycogen, mg/g tissue, wet weight
Soleus (Sol.)	6	7.28 ± 0.66
Sartorius (Sart.)	15	6.91 ± 0.26
Brachioradialis (Br.)	16	5.62 ± 0.33
P (Sol. vs. Br.)		< 0.001
P (Sol. vs. Sart.)		> 0.10
P (Sart. vs. Br.)		< 0.001

Values are means ± standard error.

[1]Soleus (red), sartorius (predominantly red), and brachioradialis (predominantly white).
[2]Number of experiments.

Table 3. Comparison of Total Glycogen Levels in Fresh and Frozen Muscle Samples of the Rhesus Monkey

Muscle	N[1]	Fresh	Frozen	Av. Difference Fresh-Frozen	P for Difference
Pectoralis (white)	6	8.48 ± 0.36	8.04 ± 0.25	+ 0.44 ± 0.25[2]	> 0.10
Gluteus maximus (white)	5	6.11 ± 0.18	6.33 ± 0.43	- 0.22 ± 0.26	> 0.10
Sartorius (red)	6	7.94 ± 0.34	7.55 ± 0.67	+ 0.39 ± 0.38	> 0.10

The fresh samples (unfrozen) were biopsied, weighed, and placed in 30% KOH within 5 minutes. The other portion was frozen in Liquid N_2 12-15 seconds after biopsy. Glycogen concentration expressed as mg/g tissue, wet wt. Values are means ± standard error, statistical analysis on basis of paired observations.

[1]Number of experiments.
[2]Standard error of the difference.

the total glycogen concentration in the fresh as compared to the frozen samples in the three muscles examined. These results suggest that the lower glycogen levels in white as compared to red muscle of the rhesus monkey were not due to a rapid decrease in the glycogen content following biopsy of the muscle from the rhesus unless such a decrease occurred within 12-15 seconds prior to freezing. The glycogen content of the gluteus maximus (white) was lower than that of the sartorius (red) while the glycogen level in the pectoralis (predominantly white) was similar to that of the sartorius.

2. TCA Soluble and Residual Glycogen Levels in Red and White Muscle of the Rhesus Monkey

The TCA soluble and residual glycogen concentrations in the sartorius and brachioradialis muscles, determined by a modification of the method of Russell and Bloom (138), are shown in Table 4. The concentrations of the TCA soluble and residual glycogen were higher in the sartorius as compared to the brachioradialis muscle, and the per cent residual glycogen was similar in the two series.

In the above determinations of the TCA soluble and residual glycogen, some thawing of the muscle samples was noted during the weighing procedure. To ascertain if thawing might alter either the TCA soluble or residual glycogen preferentially, concentrations of the two fractions in 0 time (frozen, control) samples were compared with levels in muscles frozen and then allowed to thaw at room temperature for 3 minutes. Approximately 40% of both the TCA soluble and residual glycogen disappeared during a three minute thaw of the sartorius muscle (Table 5), whereas if the muscle was held at room

Table 4. Comparison of the TCA Soluble and Residual Glycogen in the Sartorius (Red) and Brachioradialis (White) Muscles of the Rhesus Monkey.

Glycogen (mg/g)	Sartorius	Brachioradialis	Av. Dif. (Sar-Br)	P
TCA soluble	3.63 ± 0.36	2.67 ± 0.27	+0.96 ± 0.32[1]	< 0.025
Residual	2.54 ± 0.21	1.76 ± 0.10	+0.78 ± 0.19	< 0.005
Total	6.17 ± 0.56	4.43 ± 0.25		
% Residual[2]	41.2%	39.7%		

Values are means ± standard error. Glycogen expressed as mg/g tissue, wet wt. Statistical analysis on basis of paired observations, 11 experiments.

[1]Standard error of the difference.

[2]Per cent residual = residual glycogen/total glycogen x 100.

Table 5. Effect of Thawing on Levels of TCA Soluble and Residual Glycogen of the Sartorius Muscle of the Rhesus Monkey.

	Glycogen, mg/g tissue, wet wt		% Residual Glycogen
	TCA Soluble	Residual	
0 time (frozen)	3.31 ± 0.31	2.19 ± 0.18	39.8 %
3 minutes (thawed)	1.94 ± 0.18	1.33 ± 0.10	40.7 %
Av. Difference (frozen-thawed)	+ 1.37 ± 0.18[3]	+ 0.86 ± 0.18	
P[2] (frozen vs. thawed)	<0.001	<0.001	

Sartorius muscle (red) frozen in liquid N_2. One portion was allowed to thaw at 24° C for 3 minutes while the other portion remained frozen. Values are means ± standard errors, 10 experiments.

[1]Per cent residual glycogen = residual glycogen/total x 100.

[2]Statistical analysis on the basis of paired observations.

[3]Standard error of the difference.

temperature (unfrozen) for this time period no change was noted in the glycogen levels (Table 3). Furthermore, the per cent residual glycogen of the 3 minute thaw samples was similar to the 0 time control samples (about 40%) (Table 5), indicating that both glycogen fractions decreased to a similar extent during the thawing procedure. This observation is interesting since various workers have claimed that the TCA soluble is the more labile of the two glycogen fractions (138).

The effect of altering preparative procedure on levels of TCA soluble and residual glycogen was investigated by varying the time and temperature of homogenization. An increase in the duration of homogenization from 1 minute to 2 minutes (0°C) decreased the per cent residual glycogen about 6% (Table 6). Similarly, an increase in the temperature of homogenization (0° to 35°C) decreased the per cent residual glycogen by 3%. Although these alterations in the preparatory procedure significantly decreased the per cent residual glycogen from those observed under control conditions (1 minute at 0°C), the per cent residual glycogen still represented at least 33% of the total glycogen.

Since previous work has indicated that the residual glycogen is bound to protein (150) and may be extracted into the TCA medium with additional homogenization and extraction (135), the residual glycogen fraction was reextracted by two methods. In the drastic procedure the residual fraction was reextracted by rehomogenization, while in the mild procedure stirring and vortexing were employed as previously described in II B 5 b. The results (Table 7) show that after reextraction of the residual fraction by either the drastic or mild

Table 6. Effect of Various Preparatory Procedures on the Per Cent Residual Glycogen in the Sartorius Muscle of the Rhesus Monkey

	N[1]	Procedure[2]	% Residual Glycogen
	18	1 minute at 0° C	39.8 ± 1.73
		2 minutes at 0° C	33.5 ± 1.31
Av. Difference (2 min.-1 min.)			- 6.3 ± 0.01[3]
P for dif.			< 0.001
	17	1 minute at 0° C	39.2 ± 1.66
		1 minute at 35° C	36.2 ± 0.47
Av. Difference (35° C - 0° C)			- 3.0 ± 0.01
			< 0.005

Values are means ± standard errors. Residual and total glycogen expressed as mg glycogen/g tissue, wet wt. % Residual glycogen = residual glycogen/total glycogen x 100. Statistical analysis on the basis of paired observations.

[1]Number of experiments.

[2]Muscle samples homogenized for 1 or 2 minutes at 0° C or for 1 minute at 0° or 35° C analyzed for TCA soluble and residual glycogen.

[3]Standard error of the difference

Table 7. Effect of Two Different Methods of Reextraction Upon the TCA Soluble and Residual Glycogen of the Sartorius Muscle of the Rhesus Monkey

Method of Reextraction	N	TCA Soluble Fraction I	Residual	Total	$\frac{\text{Residual}}{\text{Total}} \times 100$ Before	TCA Soluble Fraction II	$\frac{\text{Residual}}{\text{Total}} \times 100$ After
Drastic[1]	12[2]	4.44 ± 0.71	3.19 ± 0.30	7.63 ± 0.98	43.8% ± 2.0	0.80 ± 0.10	33.2% ± 1.7
Mild[3]	6[4]	9.80 ± 0.87	4.77 ± 0.20	14.57 ± 1.04	33.2% ± 1.5	0.31 ± 0.03	31.0% ± 1.4

Glycogen expressed as mg glycogen/g tissue, wet wt. TCA soluble Fraction I = TCA soluble glycogen initially obtained. Residual glycogen = glycogen remaining in the residual after extraction of the TCA soluble fraction I. TCA soluble fraction II = TCA soluble glycogen obtained upon one reextraction of the residue. Values are means ± standard errors.

[1]Rehomogenization

[2]12 samples from 5 animals

[3]Stirring and vortexing

[4]6 samples from 1 animal

procedure there was a decrease in the per cent residual glycogen, and this decrease was almost 4 times greater with the drastic as compared to the mild reextraction procedure. These results indicate that the reextraction procedure influences glycogen content of the residual fraction. However, even after drastic reextraction of the residual fraction, the residual glycogen still accounted for 30% of the total glycogen, indicating that the major portion of this fraction was resistant to cold TCA extraction.

B. In Vitro Studies--TCA Soluble and Residual Glycogen

Analytically it was possible to differentiate between the TCA soluble and residual glycogen fraction. Therefore, it was of interest to compare the metabolic activities of these two fractions in red and and white muscle. Since insulin is known to increase the incorporation of glucose into glycogen, the effect of this hormone upon the glycogen fractions in red and white muscle was investigated to explain the previously observed higher incorporation of glucose into red as compared to white muscle (19).

In agreement with previous work in this laboratory (19), the incorporation of label from glucose-U-C^{14} (G-U-C^{14}) into the TCA soluble and residual glycogen in the absence of insulin was 7-11 times higher for red as compared to white muscle of the rat after a 10 minutes incubation period (Table 8). The incorporation of label into the TCA soluble glycogen was about twofold greater than that of the residual glycogen of the red muscle, while the incorporation of label into both glycogen fractions of white muscle was similar. Insulin increased the incorporation of label into both glycogen fractions in

Table 8 Comparison of Incorporation of Label from Glucose-U-C^{14} into TCA Soluble and Residual Glycogen in Red and White Muscle of the Rat in the Presence and Absence of Insulin

TCA Soluble Glycogen (10^3 cpm/g tissue wet wt)				Residual Glycogen (10^3 cpm/g tissue wet wt)			
Insulin Absent	Insulin Present	Increase with Insulin	P[1]	Insulin Absent	Insulin Present	Increase with Insulin	P[1]
			Red Muscle				
107 ± 9.2	179 ± 15.3	70.5 ± 15.1[2]	< 0.01	58.7 ± 7.6	95.3 ± 12.1	36.6 ± 8.8[2]	< 0.01
			White Muscle				
9.7 ± 3.4	25.5 ± 6.7	15.8 ± 3.6	< 0.01	8.2 ± 1.5	17.2 ± 2.2	9.0 ± 2.1	< 0.01
P[3] (R vs. W)							
< 0.001	< 0.001			< 0.001	< 0.001		

Red and white muscle fiber groups from the semimembranosus muscle of the rat were incubated 10 minutes in Krebs bicarbonate buffered medium, pH 7.4 containing 150 mg% glucose and 3.5 μc/ml glucose-U-C^{14}. Insulin (0.05 units/ml) was added to some flasks. Values are means ± standard errors. Statistical analysis on the basis of paired observations, 6 flasks from 4 animals.

[1]P for addition of insulin [2]Standard error of the difference [3]P for red versus white muscle

red and white muscle (Table 8). Although the per cent increase with insulin in the incorporation of label into the TCA soluble (162%) and residual (110%) glycogen in white muscle was approximately twofold greater than in TCA soluble (66%) and residual (62%) glycogen in red muscle, the absolute increase in glucose-C^{14} incorporation (cpm x 10^3/g muscle) was at least fourfold greater in red (70.5 cpm x 10^3/g for TCA soluble and 36.6 cpm x 10^3/g for residual glycogen) as compared to white muscle (15.8 cpm x 10^3/g for TCA soluble and 9.0 cpm x 10^3/g for residual glycogen).

C. Glucose-6-Phosphate Levels

Since the tenfold higher incorporation of glucose-C^{14} label into glycogen might be explained by variation in the pool size for precursor hexose phosphates, glucose-6-phosphate levels in red and white muscle of the rat were determined in an attempt to aid in the interpretation of results. Red and white muscle samples of the rat were analyzed for G-6-P levels (μmoles G-6-P/g tissue wet wt) as previously described (II D). No difference in G-6-P levels between the two types of muscle was observed (Table 9). (See p. 66).

D. Glucose-6-Phosphate Pools and the Effect of Insulin on These Pools

The possibility of the existence of two or more separate pools of G-6-P in voluntary skeletal muscle that differ in response to insulin was investigated by incubating muscle fiber groups in media containing either glucose-1-C^{14} (G-1-C^{14}) or glucose-6-phosphate-1-C^{14} (G-6-P-1-C^{14}) as previously described (II E 1). The results of these experiments are presented in Tables 10-15.

Table 9 Glucose-6-Phosphate Levels in Red and White Muscle of the Rat (μmoles/100 g tissue, wet wt)

Muscle		Av. Difference	P for Difference
Red[1]	White[2]	(Red - White)	
30.6 ± 3.7	24.7 ± 1.5	+ 5.9 ± 4.3[3]	> 0.10

Values are means ± standard errors. P calculated on the basis of paired observations, 13 experiments.

[1]Red muscle (deep portion of the semimembranosus and the caudofemoralis)
[2]White muscle (superficial portion of the semimembranosus)
[3]Standard error of the difference

1. $C^{14}O_2$ Assay

The incorporation of label from G-1-C^{14} into $C^{14}O_2$ was approximately 25% higher for red muscle than for white muscle of the rat in the presence or absence of insulin (Table 10). This higher incorporation of label from glucose into CO_2 in red as compared to white muscle of the rat is in agreement with previous work in this laboratory (20). Insulin increased the incorporation of label from G-1-C^{14} into $C^{14}O_2$ by red and white muscle of the rat (Table 10) and by sartorius muscle of the rhesus (Table 13). The incorporation of glucose-6-phosphate-1-C^{14} (G-6-P-1-C^{14}) into $C^{14}O_2$ for red muscle was higher than that for white muscle in the presence or absence of insulin. Insulin did not change the incorporation of label from G-6-P-1-C^{14} into $C^{14}O_2$ by either the red and white muscle of the rat or the sartorius muscle of the rhesus. Landau and Sims (89) reported similar observations in the hemidiaphragm of the rat.

Table 10. Incorporation of Label from Glucose-1-C^{14} and Glucose-6-Phosphate-1-C^{14} into CO_2 by Red and White Muscle of the Rat in the Presence and Absence of Insulin

Glucose-1-C^{14} (10^3 cpm/g tissue, wet wt)				Glucose-6-P-1-C^{14} (10^3 cpm/g tissue, wet wt)			
Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]
			Red Muscle				
67.6 ± 4.1	108 ± 9.3	+ 41.2 ± 7.2[2]	<0.005	80.8 ± 5.8	82.6 ± 7.8	+ 1.8 ± 2.8[2]	>0.10
			White Muscle				
50.8 ± 3.2	87.4 ± 5.1	+ 36.6 ± 5.3	<0.005	43.2 ± 5.3	54.2 ± 4.1	+ 11.0 ± 8.0	>0.10
P[3] <0.02	<0.05			<0.005	<0.025		

Red and white muscle fiber groups from the semimembranosus muscle of the rat were incubated 2 hours in Krebs bicarbonate buffer, pH 7.4 containing 2 mM G-6-P and 10 mM glucose and 1.0 μc/ml of either G-1-C^{14} or G-6-P-1-C^{14} and insulin (0.1 unit/ml) was added to some flasks. Values are means ± standard errors. Statistical analysis on the basis of paired observations, 5 animals.

[1]P for addition of insulin
[2]Standard error of the difference
[3]P for red versus white muscle

Table 11. Incorporation of Label from Glucose-1-C^{14} and Glucose-6-Phosphate-1-C^{14} into Lactate by Red and White Muscle of the Rat in the Presence and Absence of Insulin.

Glucose-1-C^{14} (10^3 cpm/g tissue wet wt)				Glucose-6-P-1-C^{14} (10^3 cpm/g tissue wet wt)			
Insulin Absent	Insulin Present	Av. Increase with Insulin	P^1	Insulin Absent	Insulin Present	Av. Increase with Insulin	P^1
			Red Muscle				
338 ± 13	527* ± 38	+ 186* ± 36[2]	< 0.02	501 ± 63	436 ± 43	- 64.8 ± 38.2	> 0.10
			White Muscle				
478 ± 40	669 ± 67	+ 192 ± 35[2]	< 0.01	489 ± 41	492 ± 56	+ 3.0 ± 50[2]	> 0.10
P^3 < 0.05	> 0.10*			> 0.10	>0.10		

Experimental conditions as described in Table 10. Values are means ± standard errors, P calculated on the basis of paired observations, 5 animals except, * 4 animals

[1]P for addition of insulin

[2]Standard error of the difference

[3]P for red versus white

Table 12 Incorporation of Label from Glucose-1-C^{14} and Glucose-6-Phosphate-1-C^{14} into Glycogen by Red and White Muscle of the Rat in the Presence and Absence of Insulin

Glucose-1-C^{14} (10^3 cpm/g tissue wet wt)				Glucose-6-P-1-C^{14} (10^3 cpm/g tissue wet wt)			
Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]
			Red Muscle				
254 ± 33.1	716 ± 120	462 ± 19.3[2]	< 0.001	49.6 ± 4.6	60.2 ± 2.5	10.6 ± 3.4[2]	<0.05
			White Muscle				
92.6 ± 11.9	267 ± 32.9	174 ± 25.0	< 0.005	27.4 ± 4.0	32.6 ± 4.5	5.2 ± 1.3	<0.02
P[3] <0.005	<0.02			<0.01	<0.001		

Experimental conditions as described in Table 10. Values are means ± standard errors. Statistical analysis on the basis of paired observations, 5 animals.

[1]P for additions of insulin

[2]Standard error of the difference

[3]P for red versus white

Table 13. Incorporation of Label from Glucose-1-C^{14} and Glucose-6-Phosphate-1-C^{14} into Glycogen, Lactate, and CO_2 by the Sartorius Muscle of the Rhesus Monkey in the Presence and Absence of Insulin

Product	Glucose-1-C^{14} (10^3 cpm/g tissue, wet wt)				Glucose-6-P-1-C^{14} (10^3 cpm/g tissue, wet wt)			
	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]
				Red Muscle				
Glycogen	227 ± 55	419 ± 150	+ 192 ± 99.6[2]	< 0.10	34.0 ± 5.9	37.6 ± 6.6	+ 3.60 ± 3.54[2]	> 0.10
Lactate	362 ± 28	438 ± 33	+ 76.0 ± 12.9	<0.005	264 ± 34	198 ± 20	- 66.2 ± 24.7	< 0.10
CO_2	50.8 ± 6.1	62.8 ± 8.3	+ 12.0 ± 3.0	< 0.02	39.2 ± 5.0	35.6 ± 3.3	- 3.60 ± 2.35	> 0.10

Sartorius (red) muscle fiber groups from the rhesus monkey were incubated for 2 hours in Krebs bicarbonate buffer, pH 7.4 containing 2 mM glucose-6-P or 10 mM glucose, with either 1.0 μc/ml of G-6-P-1-C^{14} or G-1-C^{14} and with and without 0.1 unit insulin/ml. Values are means ± standard error. Statistical analysis on the basis of paired observations, 5 animals.

[1]P for addition of insulin

[2]Standard error of the difference

2. Lactate-C^{14} Production

The values for lactate-C^{14} production from G-1-C^{14} in white muscle were 30% higher than for red muscle in the absence of insulin. In the presence of insulin there was one less sample which influenced the statistics (N = 4), but the individual experiments showed a greater lactate-C^{14} production by white muscle fiber groups than by red in every instance. These results are in agreement with the concept that glycolysis is more active in white than red muscle. Insulin increased incorporation of label from G-1-C^{14} into lactate-C^{14} for red and white muscle of the rat (Table 11) and for sartorius muscle of the rhesus (Table 13). There was no difference between the incorporation of label from G-6-P-1-C^{14} into lactate by the red as compared to the white muscle of the rat, although the incorporation into $C^{14}O_2$ (Table 10) was different in the two types of muscle. The amount of lactate-C^{14} produced from G-6-P-1-C^{14} substrate was not increased in the presence of insulin in red and white muscle from the rat or in sartorius muscle from the monkey. Landau and Sims also found no increase in the quantity of lactate-C^{14} produced from G-6-P-1-C^{14} in the presence of insulin by the rat hemidiaphragm (89).

3. Incorporation of Label into Glycogen

The incorporation of label from glucose-1-C^{14} into glycogen was higher in red than in white muscle in the presence and absence of insulin (Table 12). These results are in agreement with data indicating a greater total glycogen synthetase enzyme activity in red as compared to white muscle (21). In both red and white muscle the addition of insulin caused a threefold increase in the incorporation of the label

from G-1-C^{14} into glycogen as compared to a twofold increase in the labeled glycogen of the sartorius muscle of the rhesus. These results are consistent with a higher total glycogen synthetase activity in the red and white (semimembranosus) muscle of the rat as compared to the sartorius muscle of the rhesus (21). Insulin increased the incorporation of C^{14} label from G-1-C^{14} into glycogen in both red and white muscle fibers of the rat, but not into glycogen of sartorius muscle of the rhesus. However, in the rhesus series there was a greater variation in the initial glycogen levels as indicated by a larger standard error which influenced the results of the statistical analyses. However, individual experiments using the sartorius muscle fibers always showed at least a 25% increase (with an average increase of 46%, Table 13) in the incorporation of G-1-C^{14} label into glycogen with insulin present.

The incorporation of label from G-6-P-1-C^{14} into glycogen was almost twofold higher in red as compared to white muscle of the rat (Table 12). Insulin increased incorporation of C^{14} from G-6-P-1-C^{14} into glycogen of red and white muscle of the rat (Table 12) but did not effect incorporation of label into glycogen of sartorius muscle of the rhesus (Table 15). The incorporation of label from G-6-P-1-C^{14} into glycogen of rat or rhesus muscle may be accounted for by a hydrolysis of G-6-P to glucose and the subsequent incorporation of glucose into glycogen. The experiments, in which the magnitude of the possible hydrolysis of G-6-P was estimated, demonstrated that a minimum of about 7% of the counts added as the G-6-P-1-C^{14} substrate were converted to glucose counts after a 2 hour incubation of the

rat muscle fiber groups in the presence or absence of insulin.

The results of the incorporation of C^{14} label into glycogen indicated that the incorporation of both labeled substrates (glucose and glucose-6-phosphate) into glycogen was higher in red muscle as compared to white. Insulin increased the incorporation of label from G-1-C^{14} into glycogen of the red and white muscle of the rat but not into glycogen of the sartorius muscle of the rhesus. Similarly, the red and white muscle of the rat showed an increase in the incorporation of label from G-6-P-1-C^{14} into glycogen in the presence of insulin while the sartorius muscle of the rhesus did not.

4. Ratios of Incorporation

To determine if more than one G-6-P pool exists in red and white muscle of the rat and in sartorius muscle of the rhesus, the ratios of incorporation of C^{14} label from G-1-C^{14} to that from G-6-P-1-C^{14} into the products $C^{14}O_2$, lactate-C^{14}, and glycogen-C^{14} were determined. If there is only one G-6-P pool in red or white muscle and if G-6-P is assumed to be an obligatory intermediate in glucose metabolism, then the C^{14} label from either glucose or G-6-P should be incorporated in the same proportion into each of the products. If differing ratios are obtained, it would appear that more than one G-6-P pool is present.

The ratios of incorporation of the labeled substrates into glycogen were higher as compared to those of lactate and CO_2 in red and white muscle of the rat (Table 14) and in sartorius muscle of the rhesus (Table 15). In all instances the ratios for lactate as compared to CO_2 were similar ($P > 0.10$) in the absence or in the presence of insulin. Insulin increased the ratios of incorporation in most cases,

Table 14. Ratios of Incorporation of C^{14} of Glucose-1-C^{14} to that of C^{14} of Glucose-6-P-1-C^{14} into Glycogen, Lactate, and CO_2 by Red and White Muscle of the Rat in the Presence or Absence of Insulin

Ratios of Incorporation	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]
	Red Muscle				White Muscle			
Glycogen	4.4 ± 0.9	12.2 ± 2.5	+6.8 ± 2.0[2]	<0.025	2.7 ± 0.1[3]	9.3 ± 2.2	+6.6 ± 0.7	<0.005
Lactate	0.7 ± 0.1	1.3 ± 0.2	+0.6 ± 0.2	>0.10	1.0 ± 0.1	1.4 ± 0.1	+0.4 ± 0.1	<0.05
CO_2	0.8 ± 0.1	1.4 ± 0.2	+0.5 ± 0.1	<0.01	1.2 ± 0.1	1.6 ± 0.1	+0.4 ± 0.2	>0.10
P(gly.vs.lact.)	< 0.01	< 0.05[3]			<0.005[3]	< 0.025		
P (gly. vs. CO_2)	< 0.01	< 0.02			< 0.01[3]	< 0.05		
P (lact. vs. CO_2)	> 0.10	> 0.10[3]			> 0.10	> 0.10		

Experimental conditions as in Table 10. Values are means ± standard errors. Statistical analysis based upon paired observations, 5 animals.

[1]P for addition of insulin

[2]Standard error of the difference

[3]Four animals

Table 15. Ratios of Incorporation of C^{14} of Glucose-1-C^{14} to that of C^{14} of Glucose-6-Phosphate-1-C^{14} into Glycogen, Lactate, and CO_2 by the Sartorius Muscle of the Rhesus Monkey in the Presence and Absence of Insulin

Ratio of Incorporation	Insulin Absent	Insulin Present	Av. Increase with Insulin	P[1]
Glycogen	6.5 ± 0.9	10.0 ± 2.2	+ 3.5 ± 1.6[2]	< 0.10
Lactate	1.4 ± 0.2	2.3 ± 0.3	+ 0.9 ± 0.2	< 0.02
CO_2	1.3 ± 0.1	1.8 ± 0.2	+ 0.4 ± 0.1	< 0.001
P (gly. vs. lact.)	< 0.01	< 0.02		
P (gly. vs. CO_2)	< 0.005	< 0.025		
P (lact. vs. CO_2)	> 0.10	> 0.10		

Experimental conditions as in Table 13. Values are means ± standard errors. Statistical analysis on the basis of paired observations, 5 animals.

[1]P for addition of insulin

[2]Standard error of the difference

for example, for glycogen and CO_2 in red muscle of the rat, for glycogen and lactate in white muscle of the rat, and for lactate and CO_2 in the sartorius muscle of the rhesus.

Expressing the results as ratios of incorporation is useful in determining the presence of more than one G-6-P pool in muscle. However, these ratios cannot be used to estimate the incorporation of glucose or G-6-P into a product, to observe the effect of insulin on the incorporations, or to determine differences between red and white muscle. For instance, an increase in the incorporation of G-1-C^{14} as well as G-6-P-1-C^{14} might occur in the presence of insulin which in turn might cause a decrease, increase, or no effect at all in the ratio of incorporation. If a decrease were noted, it would not mean that the ratio of incorporation is nonresponsive to insulin treatment. Likewise, similar ratios for red and white muscle do not indicate the same degree of incorporation of label or the same response to insulin. For this reason, the data for the incorporation of each labeled substrate into CO_2, lactate, and glycogen and the effect of insulin on this incorporation as well as the ratios and their response to insulin were reported in this thesis.

5. Leaching Experiments

Although the ratios of incorporation seem to indicate that more than one G-6-P pool is present in muscle, it is possible that the cutting of the muscle fibers during dissection produced a second pool by leaching glycolytic enzymes into the medium. To test this possibility, muscle fibers were incubated 1 hour in medium without substrate, the tissue was removed, and G-6-P-1-C^{14} added. During an additional

2 hours incubation there was no measurable $C^{14}O_2$ by muscle fiber groups of the rat or rhesus and no appreciable lactate-C^{14} production ($+9.2 \pm 14.8 \times 10^3$ cpm/g SD, $N = 7$, $P > 0.10$ as compared to 0) by muscle fiber groups of the rat. However, production of lactate-C^{14} ($+9.7 \pm 7.9 \times 10^3$ cpm/g SD, $N = 11$, $P < 0.02$ as compared to 0) by sartorius muscle fiber groups of the rhesus was demonstrated. This lactate-C^{14} production accounted for about 2.4% of the total lactate-C^{14} produced in the presence or absence of insulin and was subtracted from the total lactate produced in each flask. Shaw and Stadie (139) also reported minimal leaching of glycolytic enzymes with rat hemidiaphragm, and Landau and Sims (89, 141) reported no $C^{14}O_2$ production and about 1.4% of the total lactate-C^{14} production due to glycolytic enzymes in the medium.

IV. Discussion

A. Preliminary Glycogen Experiments

In the rhesus monkey white muscle did not demonstrate the higher glycogen levels generally considered typical of this type of muscle as compared to red muscle (Table 2). Various reasons for this discrepancy were investigated.

1). It was thought that if the glycogen of white muscle were much more labile than that of red muscle then a rapid decrease in the glycogen content between time of biopsy and freezing of the sample might account for the lower glycogen content in control samples of white muscle. This possibility was investigated, but there was no difference in the glycogen content between samples immediately frozen (within 12-15 seconds after biopsy) and samples allowed to remain at room temperature for 2-5 minutes before processing (Table 3).

2). Next the levels of TCA soluble and residual glycogen fractions were determined in the two types of muscle. A marked difference in either fraction would be evidence that a decrease in glycogen might have occurred immediately after biopsy, and this might account for the lower glycogen in white as compared to red muscle of the rhesus. However, the per cent residual glycogen was similar for both types of muscle, indicating that the lower glycogen of white muscle was not due to a depletion in one glycogen fraction (within 12-15 seconds) as compared to the same fraction in red muscle.

3). It was also possible that some thawing had occurred during the analytical procedure for determination of TCA soluble and residual glycogen which affected one glycogen fraction more than the other.

Although thawing caused a 40% decrease in the total glycogen as compared to frozen samples analyzed without thawing (Table 5) or to nonfrozen samples (Table 3), both glycogen fractions decreased in parallel since the per cent residual was the same for frozen and thawed samples (40%) (Table 5). Even though this data did not explain the lower glycogen level in white as compared to red muscle in the rhesus, it is interesting to speculate why the glycogen in frozen and then subsequently thawed samples decreased so rapidly, while there was no change in the level of the nonfrozen samples over a 5 minute period. Recent electron microscopic studies on mouse hepatic cells demonstrated changes in the plasma membrane, mitochondria, and endoplasmic reticulum after freezing and thawing (154). These same studies indicated about a 45% increase in the enzymatic activity of glucose-6-phosphatase (a microsomal enzyme in liver). Although this enzyme is not present in skeletal muscle, it is reasonable to postulate that similar enzyme activation might occur in other glycolytic enzymes such as phosphorylase. Cori and Illingworth (32) found that freezing increased phosphorylase a because of muscular contraction associated with this process. Furthermore, Ca^{++} directly activates striated muscle phosphorylase b kinase *in vitro* (46). These observations may be of importance in explaining the 40% decrease in glycogen of thawed muscle samples. Since the sarcoplasmic reticulum is a reservoir for Ca^{++} (125), an altered sarcoplasmic reticulum might allow the release of more Ca^{++} which in turn would activate phosphorylase b kinase and account for increased glycogen breakdown.

Since the rapidity with which the sample was obtained did not

seem to explain the low glycogen of white muscle in the rhesus monkey, the explanation may lie in the suitability of the rhesus monkey for control (basal) measuerments. The rhesus monkey is a highly excitable animal, and it is known that stress causes a greater decrease in glycogen levels of white muscle than red. Therefore, the low glycogen levels in white muscle might be explained by the excited state of the animal. Various techniques were employed to obtain muscle samples without stressing the monkey (I G 1); however, we were never able to obtain a rhesus monkey with a higher glycogen level in white than in red muscle. Therefore, the comparisons between the metabolism of red and white muscle were limited to rats.

As previously discussed (I C 3) differences in solubility and extractability have often formed the basis for division of glycogen into two fractions--TCA soluble and residual. Various workers (64, 135) have claimed that only the TCA soluble glycogen exists in liver and Roe (135) reports similar findings in skeletal muscle (heart and gastrocnemius). However, other workers (75, 138) have reported differences between the two glycogen fractions in the gastrocnemius of the rat. The physiological significance of the TCA soluble and residual glycogen fractions of muscle is still questionable. Thus, before the in vitro investigation of incorporation of label from glucose into glycogen of red and white muscle was begun, the effects of variations in analytical techniques were studied. The results showed that increasing the homogenization time or temperature decreased the glycogen of the residual fraction, but even after such procedures one-third of the total glycogen was still in the residual form (Table 6). Similarly

reextraction of the residual fraction by two different procedures (Table 7) also caused a decrease (2-10%) in the residual fraction; however, about one-third of the glycogen remained in the residual form. These data indicate that it seems possible to differentiate between the two fractions of glycogen in voluntary skeletal muscle on an analytical basis. Possible metabolic differences were investigated by *in vitro* studies on the effect of insulin on glucose-C^{14} incorporation into the two glycogen fractions of red and white rat muscle.

B. In Vitro Studies--TCA Soluble and Residual Glycogen

The question of the physiological significance as well as the existence of the two fractions has been disputed. Russell and Bloom (135) found the TCA soluble glycogen to be more variable in quantity than the residual fraction. Although it is generally agreed that the TCA soluble glycogen fluctuates more under various hormonal, nutritional, and other conditions (150), there is no agreement as to which fraction is metabolically more active. Stetten, *et al.* reported a higher specific activity in skeletal muscle (unspecified) for the residual as compared to the TCA soluble glycogen (149). Kits Van Heijningen (76) found that after 7 minutes incubation there was no significant difference between the labeling of the two glycogen fractions in rat hemidiaphragm, and the addition of insulin increased incorporation into both fractions. Bocek, *et al.* (19) demonstrated that after 10 minutes incubation, there were differences between red and white muscle in the rate of incorporation of label from glucose into the two glycogen fractions.

In the present studies the incorporation of label from glucose-U-C^{14} (G-U-C^{14}) into TCA soluble and residual glycogen and the effect of insulin upon the incorporation was investigated in red and white muscle. The uptake of C^{14} label from glucose-C^{14} into the TCA soluble glycogen as compared to the residual glycogen was greater in red muscle but similar in white muscle of the rat; the total incorporation always being higher in the red muscle (Table 8). Bocek et al. (19) also found a higher incorporation of label into both glycogen fractions by red as compared to white muscle of the rat. Even though the per cent increase in incorporation was twofold greater in white as compared to red muscle for both the TCA soluble and residual glycogen upon addition of insulin, the absolute increase in incorporation was at least 4 x greater in red as compared to white muscle in the presence of insulin. Therefore, no conclusions can be drawn concerning the relative sensitivity of the two muscle types with respect to the incorporation of label from G-U-C^{14} into the TCA soluble and residual glycogen upon addition of insulin. However, since there were differences in the rates of incorporation into the two fractions, the results are consistent with the concept that the TCA soluble and residual glycogen are physiological entities (19, 76, 138).

It has been reported that differences in the molecular weight of glycogen cause differences in the rate of incorporation of label (glucose-C^{14}) into glycogen, the higher molecular weight glycogen incorporating glucose-C^{14} more rapidly than the lower molecular weight (28, 148). There may be a difference in the molecular weights of glycogen in red versus white muscle that accounts for the differences

in the incorporation of glucose into the two glycogen fractions in red as compared to white muscle in these experiments. However, no data on molecular weights of glycogen in red as compared to white muscle are available.

C. Glucose-6-Phosphate Levels in Red and White Muscle

Previous work in this laboratory (19) demonstrated that the incorporation of glucose-U-C^{14} into glycogen in red muscle was tenfold higher than in white muscle. This observation may be explained by assuming that white muscle has a higher rate of glycogenolysis or initially a larger hexose-phosphate pool than red muscle. A higher rate of glycogenolysis in white muscle might dilute the hexose-C^{14}-phosphate pool to a greater extent than in red and account for the higher incorporation of label from glucose into glycogen by red muscle. Estimates of glycogenolysis during the preparative procedure and equilibration period showed that the same amount of glycogen disappeared in both red and white muscle. This indicated that differences in the incorporation of label in red and white muscle were not the result of a greater glycogenolysis in white as compared to red muscle (19). Since no data concerning the initial pool sizes of precursor hexose phosphates in red and white muscle were available, G-6-P levels in red and white muscle were determined. It was demonstrated that the G-6-P levels are similar in both types of muscle (Table 9). Since the initial G-6-P levels are similar, the tenfold greater incorporation of label from glucose-U-C^{14} into glycogen cannot be attributed to a greater initial level of glucose-6-phosphate in white than in red muscle.

However, no data concerning G-1-P or UDPG levels in red and white muscle are available, and it is possible that differences in the initial pool sizes of these precursors might account for the differences in the uptake of C^{14} into glycogen of red as compared to white muscle. The similar levels of G-6-P in the two muscle types are one of the few parameters investigated in which red and white muscle do not show striking differences.

D. Glucose-6-Phosphate Pools and Their Response to Insulin

The possibility of two identical Embden-Meyerhof pathways in rat diaphragm that differ in cytological location and response to insulin was first reported by Shaw and Stadie (139, 140). Specifically, they reported that in one pathway labeled glucose in the medium was converted to glycogen via glucose-6-phosphate (G-6-P) and glucose-1-phosphate (G-1-P). The rate of conversion of label from glucose into glycogen as well as the levels of G-6-P and G-1-P were increased by the addition of exogenous insulin. In this same pathway when bicarbonate buffered medium was used, the label from the glucose in the medium was incorporated into lactate through G-6-P, fructose-6-phosphate (F-6-P), and 1,6-fructose diphosphate (F-D-P). Furthermore, the formation of lactate and the levels of the intermediates also were increased by insulin. Since this pathway leads to the intracellular deposition of glycogen, they proposed that it is intracellular. The insulin response exhibited by the pathway is added proof for an intracellular location since transport is considered to be a rate-limiting process for glucose metabolism in muscle.

Shaw and Stadie (140) found that the label from G-6-P was not

incorporated into glycogen or G-1-P but was incorporated into lactate via F-D-P. The incorporation of the label from G-6-P into lactate was not increased upon addition of insulin. When nonlabeled F-D-P and labeled glucose were added to the medium, labeled F-D-P was re-isolated from the medium following a 2 hour incubation period although the F-D-P of the diaphragm was unlabeled at least when the experiments were performed in phosphate buffered medium (139). Therefore, they postulated that the second pathway is situated on the surface of the cell or at some location other than the site of the first pathway. If this pathway is on the cell surface, insulin would not affect the rate of formation of lactate since glucose would probably not be rate limiting.

Recently Landau and Sims (89, 141) have investigated the problem of more than one glucose-6-phosphate pool in rat hemidiaphragm (bicarbonate buffered medium). These workers employed ratios of incorporation (previously described in III D 4) of label from glucose to that from G-6-P into CO_2, lactate, and glycogen to determine if more than one G-6-P pool was present. Their experiments suggested that CO_2 and lactate are derived from one G-6-P pool while glycogen is derived from a second pool of G-6-P to which glucose has much more access than exogenous G-6-P. Insulin did not increase incorporation of label from either G-6-P or glucose into lactate or CO_2, but insulin did increase the incorporation of the label from both substrates into glycogen. Thus they suggested that one pool is accessible to glucose and G-6-P; CO_2 and lactate are formed from this pool; and this pool is not insulin responsive. Glucose is readily accessible to the second pool to which exogenous G-6-P has a very limited access. Glycogen is formed from

this second pool which is insulin sensitive. Sims and Landau (141) also proposed a possible location of the two G-6-P pools within the muscle cell on the basis of the response of the pools to insulin. Their model of the hemidiaphragm suggests that in the absence of insulin, glucose or glucose-6-phosphate are converted primarily to lactate and CO_2, while in the presence of insulin, glucose has access to the sarcotubular system where glycogen is synthesized. This model is necessarily complicated because Sims and Landau did not find an increase with insulin in the lactate-C^{14} or $C^{14}O_2$ production from G-1-C^{14} substrate.

The results of the experiments presented in this thesis must be compared to the data of Shaw and Stadie (139, 140) and Landau and Sims (89, 141) before their significance can be discussed. The observation that the incorporation of label from glucose-1-C^{14} into glycogen in red and white muscle (Tables 12 and 13) was increased in the presence of insulin is in agreement with the results of both Shaw and Stadie (139, 140) and Landau and Sims (89. 141) in the rat hemidiaphragm. However, the incorporation of label from G-1-C^{14} into lactate and CO_2 was increased in the presence of insulin, and these results are not in agreement with Landau and Sims, who found that lactate and CO_2 production from glucose were not increased by insulin. The present results do agree with those of Shaw and Stadie (140), who noted an increased lactate production from glucose with insulin when bicarbonate buffered medium was used.

In these experiments and in those of Landau and Sims (89, 141)

there was an incorporation of label from G-6-P into glycogen that was increased by insulin. In the present studies the total G-1-C^{14} obtained from the hydrolysis of G-6-P-1-C^{14} to G-1-C^{14} can account for the observed incorporation of label from G-6-P-1-C^{14} into glycogen-C^{14} by red and white muscle of the rat (Table 12) and sartorius muscle of the rhesus (Table 13). Probably hydrolysis of G-6-P accounts for a major portion if not all of the label appearing in glycogen. Hydrolysis did occur, and the data showed that the label of G-6-P-1-C^{14} was incorporated much less readily than that of G-1-C^{14} into glycogen-C^{14}. Furthermore, insulin increased the incorporation of label from G-6-P into glycogen. The work of Shaw and Stadie and Landau and Sims as well as the results in this thesis all agree on one important point--that is, the incorporation of label from G-6-P into lactate was not increased by insulin. Our experiments and those of Landau and Sims also showed that the incorporation of label from G-6-P into CO_2 was not increased by insulin. Although the G-6-P-1-C^{14} incorporation into lactate-C^{14} and $C^{14}O_2$ was not increased by insulin, probably G-1-C^{14} from the hydrolysis of G-6-P-1-C^{14} is incorporated into these products. However, any increase in the incorporation of label from the hydrolyzed G-6-P into CO_2 and lactate would not be readily detected because the label from exogenous G-6-P was readily accessible to this pathway, while it was not readily accessible to the pathway to glycogen.

As previously mentioned (III D 4), if G-6-P is assumed to be an obligatory intermediate in the metabolism of glucose and if only a single pool of G-6-P exists, then the incubation of muscle with either

labeled glucose or G-6-P should yield similar ratios for the incorporation of the label from glucose to that of the label from G-6-P into the products glycogen, lactate, and CO_2. However, differing ratios would indicate the existence of two or more separate pools of G-6-P. Since higher ratios were obtained (Tables 14 and 15, III D) for glycogen as compared to lactate and CO_2, it is concluded that probably at least two separate G-6-P pools exist in the red and white muscle of the rat and in the sartorius muscle (red) of the rhesus monkey. Thus glycogen is derived primarily from one G-6-P pool in which exogenous G-6-P has a limited access compared to exogeneous glucose, while lactate and CO_2 are derived from another common pool in which G-6-P and glucose have about the same access. Such conclusions agree with those of Landau and Sims with respect to G-6-P pools in the rat diaphragm.

Other explanations for the differing ratios must also be discussed. First, it might be argued that if G-6-P-1-C^{14} is hydrolyzed to G-1-C^{14}, then the appearance of label in CO_2, lactate,and glycogen is due to the subsequent metabolism of the G-1-C^{14} formed. Since hydrolysis would tend to favor similar ratios (if only one G-6-P pool were present) rather than dissimilar ones, this idea may be disregarded. As previously discussed the amount of hydrolysis noted in these experiments can account for the incorporation of G-6-P into glycogen, as well as the insulin effect on this incorporation. Similarly, hydrolysis could also account for some incorporation of G-6-P into lactate and CO_2 in the work presented in this thesis and in the work of Landau and Sims (89, 141). The two different G-6-P pools might also be explained

by a leaching of glycolytic enzymes into the medium of a system. Since the results indicate no significant production of lactate or CO_2 due to leaching, it is concluded that the differing ratios were not the result of a leaching phenomena or a second G-6-P pool in the medium. Landau and Sims (89, 141) found no $C^{14}O_2$ production and negligible lactate-C^{14} production due to leaching; Shaw and Stadie (139, 140) found only minimal lactate production (rat diaphragm).

If the data in this thesis for the incorporation of the label from glucose and G-6-P into CO_2, lactate, and glycogen as well as the ratios of incorporation into these products are carefully studied and integrated, it becomes clear that two Embden-Meyerhof pathways might be involved in voluntary skeletal muscle which are similar in many respects to those proposed by Shaw and Stadie (139, 140). Figure 4 illustrates the two pathways.

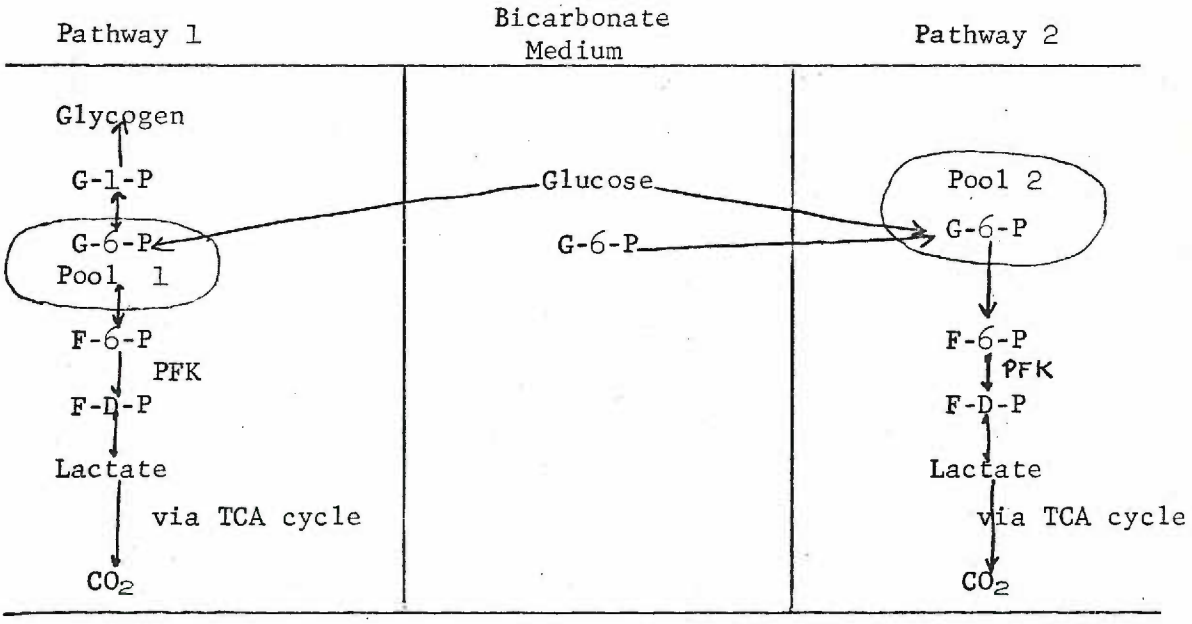


Fig. 4 Diagram of the Two Proposed Embden-Meyerhof Pathways

In the first proposed pathway, the label of glucose is incorporated into glycogen, and this incorporation is responsive to insulin. Although the data in this thesis does not offer any direct evidence, it is likely that in this same pathway the label from glucose (via G-6-P pool 1) is incorporated into lactate and CO_2. Shaw and Stadie (139) did find that in phosphate media containing labeled glucose and nonlabeled 1,6-fructose diphosphate (F-D-P) that after a 2 hour incubation labeled F-D-P was found in the medium but not in the extract of the diaphragm. Later (140) they demonstrated that the enzyme phosphofructokinase (PFK) was inactive in phosphate medium but active in bicarbonate medium, indicating that another pathway was operative when the PFK enzyme in one pathway was inactive. This evidence for two pathways is important in the interpretation of the data in this thesis because it suggests that the label from glucose is incorporated into lactate and CO_2 as well as glycogen via pathway 1.

Presumably the glucose in the medium would have access to both pathways and thus in pathway 2 (Fig. 4) the incorporation of label from glucose into lactate and CO_2 should be increased by insulin since transport across the cell membrane is assumed to be the primary rate-limiting reaction in this pathway. Also in the second proposed pathway, the label from G-6-P is incorporated into lactate and CO_2, but in this instance the addition of insulin does not increase the incorporation of label into either product.

The information gained from the ratios of incorporation provides evidence for two separate pathways since these results indicate that

glycogen is derived from a different G-6-P pool than lactate and CO_2. Thus the G-6-P pool from which glycogen-C^{14} is derived with G-1-C^{14} as substrate is located on the first pathway and the results show that this pool is not readily accessible to G-6-P from the medium. The G-6-P pool from which lactate-C^{14} and $C^{14}O_2$ are derived with G-6-P-1-C^{14} as substrate is on the second pathway and G-6-P in the medium has access to this pool. As previously mentioned it is reasonable to assume that some lactate-C^{14} and $C^{14}O_2$ are derived from the same G-6-P pool (pathway 1) as glycogen-C^{14}. When G-1-C^{14} is substrate it is not possible to demonstrate how much lactate-C^{14} and $C^{14}O_2$ are derived from pathway 1 as compared to 2.

Since insulin increases the rate of transport of glucose across the cell membrane (108), stimulates the hexokinase enzyme (26, 27, 162), increases the activity of glycogen synthetase enzyme (35, 160), and stimulates glycolysis (112, 113) and the TCA cycle (65), it is not surprising to find an increased incorporation of label from G-1-C^{14} substrate into glycogen, lactate, and CO_2. It is more difficult to explain the fact that the label from G-6-P substrate incorporated into CO_2 and lactate (Pathway 2) is not increased by insulin. Although such a suggestion is mere speculation, the results in this thesis are compatible with the hypothesis that the transport of a phosphorylated intermediate such as G-6-P is not necessarily via the same carrier mechanism as glucose. If a different transport system is involved, then there is no reason to assume that G-6-P would be responsive to insulin.

The data in this thesis (Tables 10-15) provides evidence for at

least two separate pools of G-6-P in the red and white muscle of the rat and in the sartorius muscle of the rhesus monkey. There is also evidence for two different Embden-Meyerhof pathways but no definite conclusions can as yet be drawn from the data in regard to this problem. Critical to these conclusions and suggestions is the assumption that G-6-P is an obligatory intermediate in glucose metabolism. If this assumption is not correct, the data might be interpreted to indicate that glucose is converted to glycogen by an insulin responsive pathway not involving G-6-P as has been proposed by Beloff-Chain et al. (15). Leloir (94) has briefly discussed alternate pathways for the synthesis of glycogen not involving G-6-P and concluded that further investigation is necessary to elucidate the problem, but at the present time there does not appear to be any major pathway for the synthesis of glycogen not involving G-6-P.

E. Comparison of the Incorporation of Label from Glucose-1-C^{14} and Glucose-6-Phosphate-1-C^{14} into CO_2, Lactate and Glycogen in Red and White Muscle

At the end of 2 hours incubation the incorporation of label from G-1-C^{14} into CO_2 (Table 10) and glycogen (Table 12) is higher for red muscle than for white, while the incorporation of label from G-1-C^{14} into lactate (Table 11) is higher for white muscle than for red. The results are in agreement with previous work in this laboratory (19,20). Insulin increased incorporation in all instances. The incorporation of label from G-6-P-1-C^{14} into CO_2 and glycogen is greater for red than white muscle while an equal incorporation into lactate for both types of muscle is noted.

Citric acid cycle activity has been generally assumed to be higher

in red than in white muscle. Furthermore, histochemical studies have demonstrated that citric acid cycle enzyme activity is higher in red muscle than in white (42). However, in the present experiments, the higher incorporation of label from glucose or G-6-P into CO_2 in red muscle could be accounted for by the higher specific activity of the lactate presented to the TCA cycle in red than in white muscle. Previous work in this laboratory has indicated that the specific activities of CO_2, lactate, and glycogen after a 2 hour incubation period are higher in red than white muscle of the rat (20). Therefore, no conclusions regarding the activities of the citric acid cycle in red as compared to white muscle can be made from the data presented in this thesis.

The fact that white muscle produces more lactate under resting conditions as compared to red muscle has been used as evidence that glycolysis is higher in white than in red muscle. The results in this thesis show that the production of lactate from labeled glucose is higher in white than in red muscle. The measurement of lactate production at any one time also must reflect the rate of utilization of lactate via the TCA cycle. For instance, if red muscle oxidizes more lactate via the TCA cycle in a specified time than white muscle does, the rate of glycolysis, as measured by the lactate-C^{14} production might appear to be lower for red muscle, and glycolytic activity would seem to be more active in white muscle than in red. However, in these experiments this does not appear to be the case. The amount of lactate produced plus the amount oxidized by the TCA cycle can be estimated from the data and used as a measurement of glycolytic activity in red

as compared to white muscle. Red muscle produces a total of 406 cpm/g 10^3 of lactate-C^{14} plus $C^{14}O_2$ and white muscle 529 cpm/g 10^3 (Tables 10 and 11). Therefore it appears that glycolysis is more active in white muscle under the conditions of this experiment. We have no adequate explanation for the fact that lactate-C^{14} production from labeled G-6-P in red and white muscle is not significantly different and the sum of lactate-C^{14} plus $C^{14}O_2$ production is very close, less than a 10% difference, in red and white muscle. The difference between the rates of glycolysis of glucose and G-6-P in red as compared to white muscle cannot be adequately explained at the present time. Glucose uptake is higher in red than in white muscle (20), and similarly the hexokinase activity in red muscle is about 8 x higher than in white muscle of the rabbit (123).

A higher incorporation of label from glucose into glycogen of red as compared to white muscle in the presence or absence of insulin is consistent with the higher total glycogen synthetase activity in red muscle (21). Since there is some hydrolysis of G-6-P-1-C^{14} to G-1-C^{14} (a minimum of about 7%) in these experiments, the subsequent metabolism of this G-1-C^{14} to glycogen probably accounts for the greater "apparent" incorporation of label from G-6-P into glycogen in red as compared to white muscle as well as the incorporation of exogeneous G-6-P into glycogen in red or white muscle.

V. Summary and Conclusions

1. Preliminary investigation on sartorius (red) and brachioradialis (white) muscle of the rhesus monkey revealed a higher glycogen level in red as compared to white muscle under resting conditions. These results were not in agreement with previous results in the rat and other mammals, which indicate a higher glycogen level in white than in red muscle.

2. Although glycogen determinations were performed under a variety of conditions and various techniques were employed to obtain muscle samples without stressing the monkey, it was not possible to obtain a rhesus monkey with a higher glycogen level in white than in red muscle. Thus, the *in vitro* studies were continued using red and white muscle of the rat and sartorius (red) muscle of the rhesus.

3. The effects of insulin on the carbohydrate metabolism of red and white muscle from the hind limbs of the rat and of red (sartorius) muscle of the rhesus monkey were investigated.

4. Incorporation of label from glucose-C^{14} into TCA soluble as compared to residual glycogen from red muscle was higher, but the incorporation into both glycogen fractions of white muscle was similar, the incorporation of label into red muscle always being higher.

5. Insulin increased the incorporation of label from glucose-C^{14} into both glycogen fractions in red and white muscle of the rat. The per cent increase was greater for white, while the absolute increase was greater for red muscle. Although variations in analytical technique caused a small decrease in the residual glycogen, these studies are consistent with the concept that the TCA soluble and residual glycogen

are physiological entities.

6. No differences were found in the glucose-6-phosphate levels in red as compared to white muscle. Therefore, differences in incorporation of glucose into the glycogen of red and white muscle can not be explained by differences in initial G-6-P levels.

7. In vitro studies provide evidence for at least two glucose-6-P pools in red and white muscle of the rat and in sartorius muscle of the rhesus.

8. Insulin increased the incorporation of label from glucose into glycogen, lactate, and CO_2 but did not increase the incorporation of label from glucose-6-phosphate into lactate and CO_2.

9. Two separate Embden-Meyerhof pathways were proposed on the basis of the two separate glucose-6-phosphate pools and differences in insulin response between glucose or glucose-6-phosphate as substrates. Glucose-6-phosphate had access to one pathway while it had a much more limited access to the other.

10. The incorporation of label from glucose and glucose-6-phosphate into glycogen and CO_2 was higher for red than white muscle of the rat. Lactate-C^{14} production from G-1-C^{14} was higher in white than in red muscle. However, there was no difference in the lactate-C^{14} production from glucose-6-phosphate in red and white muscle. Since lactate-C^{14} production plus $C^{14}O_2$ production from G-1-C^{14} was higher in white as compared to red muscle, it appears that glycolysis is more active in white muscle.

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