THE EFFECTS OF NEMBUTAL AND PROCAINE ANESTHESIA, AND BIOPSY TECHNIQUE ON MUSCLE HIGH ENERGY PHOSPHATE LEVELS OF NORMAL DOGS

by

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PREFACE

Biopsy and analysis of the high energy phosphate fractions of muscle in health and disease has become a promising research approach to a better understanding of muscle pathology. Several variable factors present problems in interpretation and comparison of data obtained in this way.

The factors investigated in this study may be divided into three general categories:

1) selection of the anesthetic agent, and the duration of anesthesia used in obtaining the biopsy specimen

2) the selection of representative muscles to be used for sampling

3) the type of biopsy technique used in obtaining the muscle sample

The anesthetic agents investigated in this project were sodium pentobarbital (Nembutal), a barbiturate producing anesthesia of intermediate duration, and procaine hydrochloride (Novocaine), a local anesthetic agent. Biopsies were taken at regularly spaced intervals from dogs anesthetized with sodium pentobarbital, and also in animals in which procaine hydrochloride infiltration was used to produce anesthesia.

An effort was made to utilize as many different muscles as possible for comparison in this project. Muscles were arbitrarily divided into two groups, "active" and "holding". The "active" muscles include muscles which are ordinarily concerned with short bursts of tetanic activity, such as Quadriceps Femoris, Flexor Digitorio, Gastrocnemius, and Triceps. The muscles designated as "holding" muscles include those ordinarily concerned with the maintenance of posture, such as Rectus Abdominis, Internal Abdominal Oblique, and External Abdominal Oblique.

Biopsy techniques were investigated by a comparison of the standard surgical technique, in which the specimen was dissected free from the adjacent muscle mass and subsequently frozen, and the "in situ" technique, in which the specimen was frozen in situ and later separated from the main body of the muscle after preservation was assured.

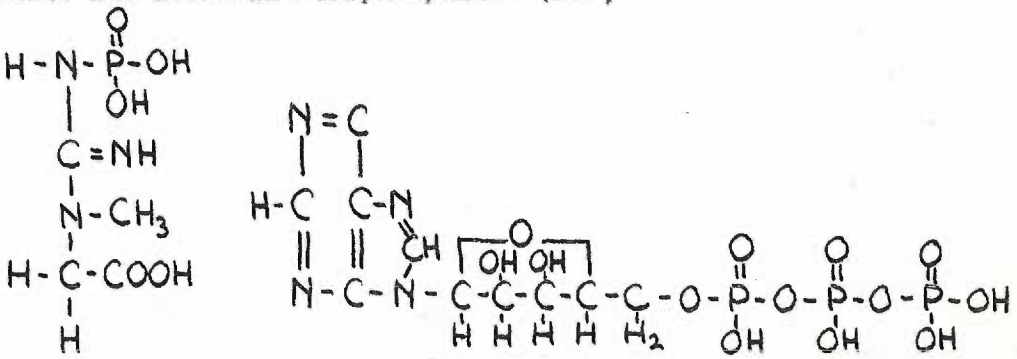
INTRODUCTION

1. The Biochemistry of Skeletal Muscle

A. Energy Metabolism in Muscle

The direct sources of energy utilized for muscle contraction are the high energy phosphate compounds. The energy stored, and released by the splitting of these compounds is provided by the two basic energy-producing mechanisms in the cell, the glycolytic cycle and the tri-carboxylic acid cycle (respiration).

Energy is stored and utilized by a unique mechanism involving the interrelationship of two important organic phosphate compounds, creatine phosphate and adenosine triphosphate. (ATP)



CREATINE PHOSPHATE ADENOSINE TRIPHOSPHATE

These substances exist in equilibrium with each other in the cell; creatine phosphate serves as the reservoir of high energy bonds, and transfers them to ADP forming ATP, which is the immediate source of energy for muscle contraction. Hydrolysis of ATP, which occurs coincident with contraction, yields adenosine diphosphate (ADP) and inorganic phosphate (IP). As the high energy bonds of ATP are depleted by contraction and other metabolic work, ADP is rephosphorylated by creatine phosphate to reform ATP. These compounds thus maintain the follow-

ing relationships with one another.

(1) $ATP + HOH \longrightarrow ADP + [\sim PO_4 \rightarrow IP + Energy]$

(2) $CrP + ADP \rightleftharpoons Cr + ATP$

(3) $IP + Energy \text{ from } \begin{cases} \text{glycolysis} \\ \text{oxidation} \end{cases} + ADP \longrightarrow ATP$

Reaction (2) is reversible, and creatine phosphate reserves are replenished from ATP when it is available, during the resting phase. During prolonged activity, ATP is continually replenished at the expense of creatine phosphate, so that the first indication of high energy bond depletion is reflected in a decrease in the level of creatine phosphate. ATP, in turn, is produced through a series of reactions, glycolysis and oxidation, from inorganic phosphate and ADP. (Reaction (3))

The concept of the high energy bond as a source of energy for contraction is a relatively recent development. Early workers believed that lactic acid formed from the degradation of glycogen was the energy-producing reaction responsible for contraction. Meyerhof (1), in early investigations of the thermodynamics involved in contraction, concluded that lactic acid alone could not account for the quantity of energy concerned in contraction. Hence, another source of energy was sought.

In 1927, Eggleton and Eggleton (2) discovered a labile phosphorus compound present in the trichloroacetic acid extract of muscle which decomposed slowly under the conditions of the ordinary phosphate determination. This substance was called "phosphagen" and shortly after, was identified by Fiske and SubbaRow (3) as creatine phosphate. At about the same time, Lohmann (4) identified another previously unrecognized component of the muscle phosphates, by subjecting the trichloroacetic acid

extract to acid hydrolysis (1 N.hydrochloric acid) for seven minutes. In 1929, Lohmann and Fiske and Subbarow (5) identified this substance as adenosine triphosphate.

The final blow to the lactic acid theory came when Lundsgaard (6) demonstrated that frog muscle poisoned with iodoacetate was capable, after death, of performing up to one hundred contractions without any increase in lactic acid. This proved that anaerobic activity could occur without accumulation of lactic acid. Lundsgaard showed in addition that muscle contraction continues until dephosphorylation of creatine phosphate is complete, and that high energy bonds can be provided by glycolysis.

Lohmann (7) showed that creatine phosphate serves as the reservoir of phosphate bonds, demonstrating that in muscle extracts, creatine phosphate is dephosphorylated only in the presence of ADP or AMP (adenosine monophosphate).

Lipmann (8) developed the concept of the high energy phosphate bond, and early investigators, using acid and enzyme hydrolysis, showed the energy value of this bond to be approximately 11,000 calories per mole. More recent investigations (9,10) have shown that true values are much lower than this, from 5,300 to 7,900 calories per mole. These values are still considerably higher than the energies of phosphate bonds of other biologically important phosphates. (11)

The early workers who believed that energy was provided from the breakdown of glycogen to lactic acid were partially correct. Glycolysis provides some energy in the form of ATP as shown in Figure 1. Under aerobic and anaerobic conditions, glycogen is broken down to glucose-1-phosphate by phosphorylase; glucose-1-phosphate then passes through a

series of chemical transformations to fructose-1-6-diphosphate, with the expenditure of one ATP. (reaction 4) Next, fructose-1-6-diphosphate is cleaved to form two triose phosphate molecules, 3-phosphoglyceric aldehyde and dihydroxyacetone phosphate. These two substances are in equilibrium with one another, and are interconverted by triose phosphate isomerase. During the conversion of 3-phosphoglyceric aldehyde to 3-phosphoglyceric acid, an intermediate product, 1-3-diphosphoglyceric acid is formed, which, upon reacting with ADP, yields ATP. (reaction 7) During this process, 2 hydrogens are released which combine with diphosphopyridine nucleotide (DPN) to convert it to its reduced form, DPN.2H. Under anaerobic conditions, DPN.2H is oxidized to DPN by pyruvic acid, the main end-product of glycolysis; by this reaction pyruvic acid is converted to lactic acid. (reaction 12)

3-phosphoglyceric acid forms 2-phosphopyruvic acid, enol form, which reacts with ADP to form ATP and enol-pyruvic acid. The ultimate end-product of this chain of reactions is lactic acid. Under aerobic conditions, the DPN.2H formed during reactions 7 and 10 is oxidized through the flavoprotein, cytochrome, and cytochrome oxidase systems, to form water and DPN, with the production of 3 ATP. The DPN is then available for further reduction by hydrogen. Lactic acid does not accumulate unless the oxidative processes fail to keep pace with the glycolytic reactions, and pyruvic acid must react with DPN.2H to form lactic acid.

During glycolysis, then, 10 ATP are produced for each C-6 unit of glycogen metabolized. One ATP is used in the conversion of fructose-6-phosphate to fructose-1-6-diphosphate, leaving a net gain of 9 ATP per C-6 unit of glycogen.

The oxidative cycle usually produces the greatest fraction of ATP in the muscle cell. (Figure 11) Pyruvic acid is the chief end-product of glycolysis, and the oxidative cycle consists of the oxidation of pyruvic acid with the production of utilizable energy as ATP. Pyruvic acid is decarboxylated to acetate, which is introduced into the cycle by the action of Coenzyme A. Acetyl-CoA condenses with oxaloacetic acid to form citryl-CoA and citric acid which proceeds on around the cycle. The final end-product of the cycle is oxaloacetic acid, which is available for further condensations. Some pyruvic acid is carboxylated and thus enters the cycle as oxaloacetate. ATP is formed from the oxidation-reduction reactions occurring incidental to the oxidation of hydrogen atoms from various members of the cycle. The oxidative condensation of pyruvic acid and oxaloacetic acid is the source of 3 ATP; in reaction 6 there is a loss of 2H, which is taken up by triphosphopyridine nucleotide, and leads to the production of 3 ATP. The conversion of alpha-ketoglutaric acid to succinic acid accounts for 4 ATP. 2 ATP are produced by the conversion of succinic acid to fumaric acid. And finally, the 2H lost in the conversion of malic acid to oxaloacetic acid react with DPN and lead to the production of 3 ATP. In summary, each mole of triose entering the oxidative cycle is responsible for the production of 20 ATP. This means that 40 ATP are produced for each mole of hexose metabolized. If the hexose is derived from glycogen, a net gain of 39 ATP results, since 1 ATP is required for phosphorylation, as described previously. If the hexose is free and not derived from glycogen, 2 ATP are lost by phosphorylation of hexose, leaving a net gain of 38 ATP. The oxidative cycle thus yields about four times more energy per glucose molecule than the glycolytic system. In addition to pyru-

FIGURE I

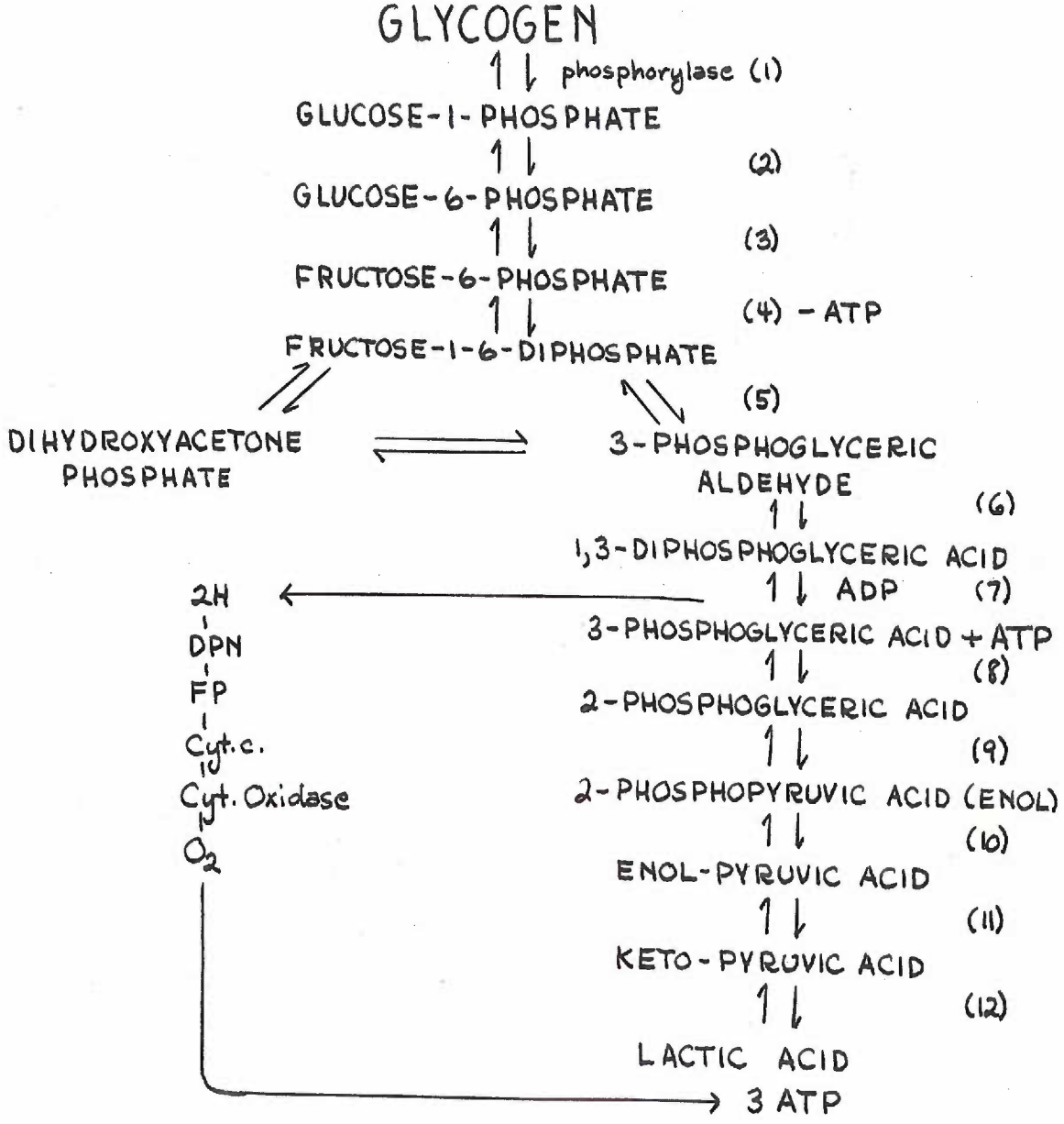
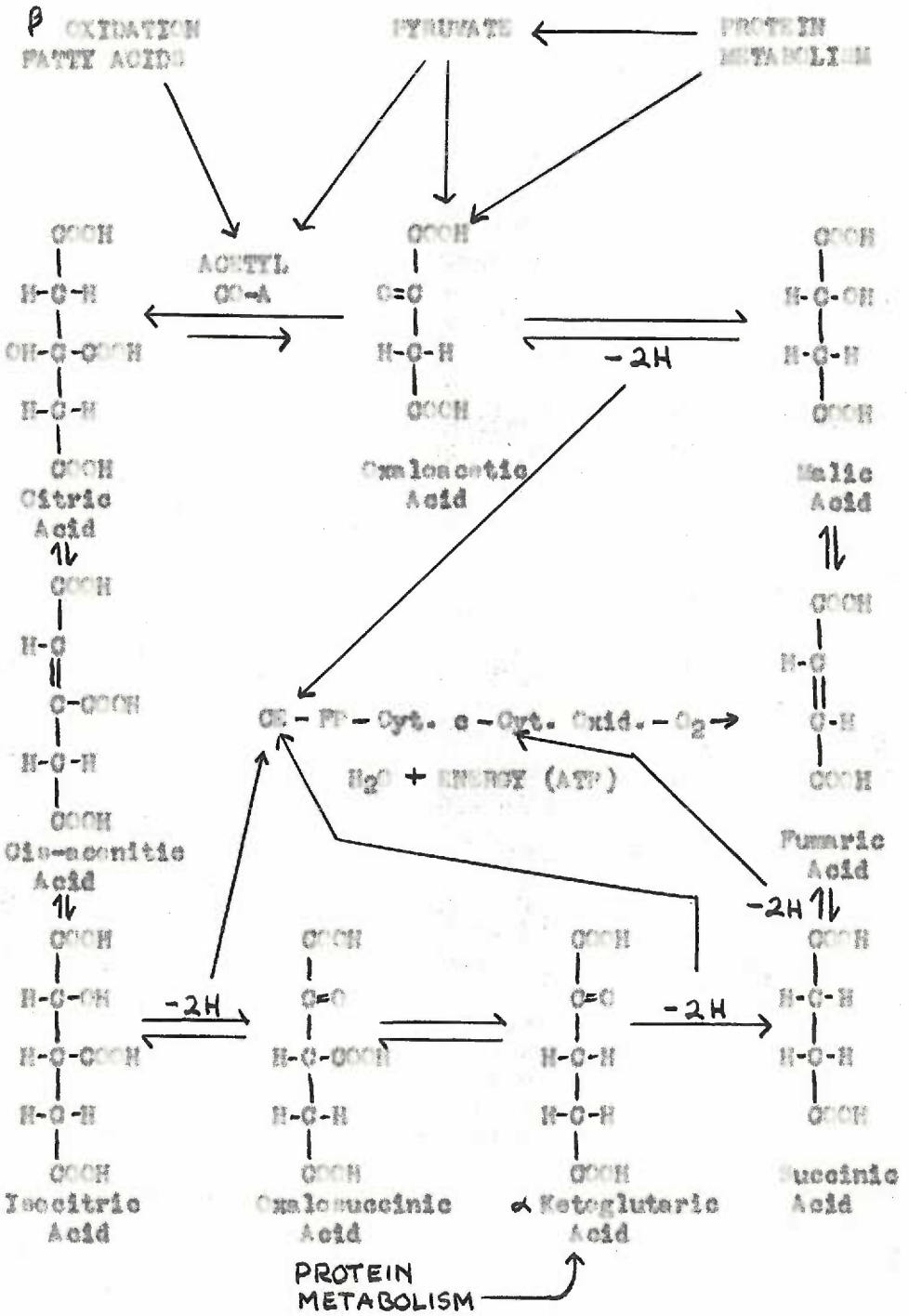

GLYCOGEN
phosphorylase (1)
GLUCOSE-1-PHOSPHATE
(2)
GLUCOSE-6-PHOSPHATE
(3)
FRUCTOSE-6-PHOSPHATE
(4) - ATP
FRUCTOSE-1-6-DIPHOSPHATE
(5)
DIHYDROXYACETONE PHOSPHATE
3-PHOSPHOGLYCERIC ALDEHYDE
(6)
1,3-DIPHOSPHOGLYCERIC ACID
ADP
(7)
3-PHOSPHOGLYCERIC ACID + ATP
(8)
2-PHOSPHOGLYCERIC ACID
(9)
2-PHOSPHOPYRUVIC ACID (ENOL)
(10)
ENOL-PYRUVIC ACID
(11)
KETO-PYRUVIC ACID
(12)
LACTIC ACID
2H
DPN
FP
Cyt. c.
Cyt. Oxidase
O2
3 ATP

FIGURE II

THE TRI-CARBOXYLIC ACID CYCLE



vate, products of beta oxidation of fatty acids, and of the oxidation of amino acids enter the tricarboxylic acid cycle as shown in Figure II. These represent additional sources of ATP, exclusive of hexose metabolism. Lehninger et al (12) have shown that the beta-oxidation of fatty acids is also productive of ATP.

B. Muscle Proteins, Contraction, and Adenosinetriphosphatase

While ATP provides the direct energy necessary to produce muscle contraction, the proteins of muscle are responsible for the mechanical act of contraction. The entire process is derived from an interaction of ATP and these proteins.

The two important proteins involved in this activity are actin and myosin. Weber (13) first postulated that myosin was the main constituent of the contractile parts of muscle fibrils. Myosin is a long, thin, molecule, and is composed of two elements, the "meromyosins". Szent-Gyorgi (14) has shown that each myosin molecule is composed of six meromyosins, two heavy molecules, called H-meromyosins, and four lighter ones, called L-meromyosins. These appear to be arranged in a special way, with the four L-meromyosins in a line, end to end, and one H-meromyosin at each end of the chain. They are bound together in this arrangement by peptide bonds. The two meromyosins have been separated from one another, and possess different properties. H-meromyosin is the more active of the two, and possesses the ability to interact with actin, as well as the ability to split high energy bonds from ATP. The phenomenon of contraction requires that myosin be present in its entirety, with both meromyosin elements in their proper arrangement.

Actin is a "globular" molecule; electron microscopy reveals that actin is either round or very nearly so. Actin exists in two forms,

which are designated the F (fibrous) and G (globular) forms. The act of contraction appears to be related to the polymerization of actin from the G to the F form. This polymerization is dependent on the presence of ATP and magnesium ion. Actin binds ATP, molecule for molecule, and dephosphorylates it during polymerization. Myosin causes instantaneous polymerization of actin from the G to the F form.

Actomyosin is the product of combination of actin and myosin. It appears that myosin is dissociated into actin and myosin during rest, and combined during contraction. ATP is necessary for the dissociation of actomyosin; this requirement is compatible with the knowledge that ATP, required for contraction, is also a substance necessary for relaxation of skeletal muscle. (15) The resulting form of actomyosin depends not only on ATP, but also on the state of ionic balance existing in the cell. The details of this relationship are beyond the scope of this discussion, but in general, it may be said that magnesium and potassium ions are of great importance in the activity of ATP and the state of viscosity of the protein-ATP complex, respectively. The conventional view of muscle contraction has included the folding or shortening of the myosin molecule as the fundamental process. The contraction cycle may be represented by the following scheme:

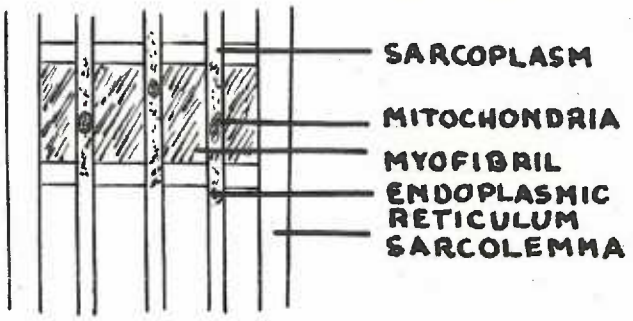
RELAXATION CONTRACTION

Reference was made earlier to the ability of H-meromyosin to split the high energy bond of ATP with the production of free energy. This

process is accomplished by the enzyme, adenosinetriphosphatase (ATPase). In 1939, Engelhardt and Ljubimova (16) demonstrated that myosin possesses ATPase activity and in recent years it has been established that ATPase and myosin are identical. Recent work (17) has shown that muscle contains two ATPases, the myofibrillar, and the granular. The myofibrillar, which is apparently identical with myosin, is responsible for the majority of ATPase activity in the cell, and is assumed to be intimately concerned with contraction. The granular ATPase is located in the interstitial granules, which include the mitochondria. It accounts for 10-25% of the ATPase activity. Investigations have shown that mitochondrial ATPase in muscle is similar to ATPase in other tissues.

C. Cellular Organization and Biochemical Activities in Muscle

In considering the possible effects of anesthetic or other external agents on the biochemical relations of muscle, it is of importance to note the difference in structure and corresponding differences in chemical requirements of different muscles. Szent-Gyorgi (14) has pointed out the parallel between muscle function and biochemical structure. Active muscles such as the flight muscle of pigeon and heart muscles utilize oxidative metabolism primarily for ATP maintenance. These muscles contain a low level of creatine phosphate. Skeletal muscles which are engaged in postural maintenance depend more on the glycolytic system for ATP maintenance, and usually have a higher creatine phosphate level. These biochemical differences are reflected in the constitution of the respective muscle cells. The following is a diagrammatic representation of the arrangement of components in the muscle cell:



The granules, which contain mitochondria (17,18) vary in size and number with different types of muscle. It has been established (18,19) that mitochondria are responsible for oxidation and phosphorylation, and that the preservation of mitochondrial structure is dependent in turn on a high energy phosphate reserve. It is not surprising, therefore, that a close relationship has been found between mitochondrial content and the oxidative ability of muscles. Those muscles which depend on oxidation for ATP maintenance have more mitochondria of higher oxidative activity than those which rely primarily on glycolysis. The latter consist of pale fibers poor in mitochondria with a low oxidative activity. It is evident that indiscriminate selection of muscle samples for high energy phosphate analysis might exert considerable influence on the values obtained. For example, this has been borne out by the observation that heart muscle (14) contains much lower levels of creatine phosphate than various skeletal muscles. In considering serial biopsy techniques in human beings, in which muscle samples are taken from various muscles at different times, the implication that the muscles selected must be comparable is obvious.

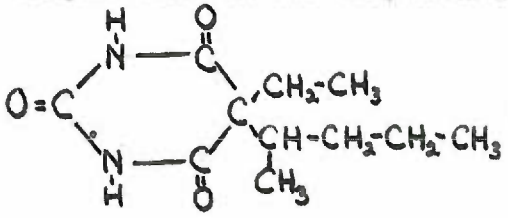
II Modes of Action and Metabolic Effects of Anesthetics

A. Sodium Pentobarbital and Related Barbiturates

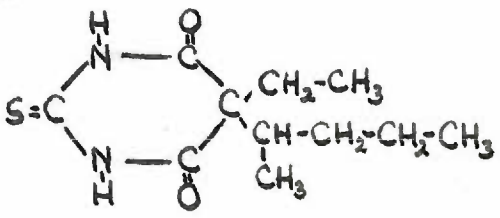
1. Chemical Composition, Distribution, and Cellular Sites of Action

Sodium pentobarbital is the sodium salt of the ethyl, 1-methyl-butyl derivative of barbituric acid. (20) It has long been used as a hypnotic and in sufficient doses is capable of producing a surgical level of anesthesia. (21) It is of interest to investigate its possible effects on the high energy phosphate relationships of muscle for three reasons.

(1) Sodium pentobarbital is the analogue of thiopental, a widely used surgical anesthetic, and differs chemically from the latter only in the replacement of an oxygen atom for a sulphur atom in the C2 position.



PENTOBARBITAL



THIOPENTAL

(2) Investigations have shown that the barbiturates are widely distributed throughout body tissues when administered in anesthetic doses, and are specifically present in skeletal muscle. (22)

(3) Recent investigations into the possible sites of action of barbiturates in the cell indicate that they probably influence the high energy phosphate levels of the tissues in which

they are present. (23)

Determination of the distribution of barbiturates in the animal body has been delayed by one major difficulty: the lack of a highly specific method by which the individual barbiturates may be separated from their metabolites. In 1947 Brodie and associates (24) developed a method of comparative partition ratios, which, when applied to organic extraction procedures, made possible the more accurate determination of these compounds in various body tissues.

The biological distribution of a drug is determined not only by its ability to penetrate capillary and cell membranes, but also by the extent to which it is bound by tissue proteins, nucleic acids, and other specific tissue elements, such as fat. According to numerous analyses of body tissues following barbiturate administration, barbiturates probably enter all body cells. The extent of binding to proteins differs from one derivative to another, and is at least partially correlated with the length of the side chains, R1 and R2. (25) Binding is also influenced by other chemical differences; the thiobarbiturates are bound to a greater extent to human serum albumin than their oxygen analogues. For example, under the same conditions, 40% of pentobarbital and 65% of thiopental are bound. Pentobarbital also differs from thiopental in its distribution in body fat. It is less concentrated in fat depots (26) and most workers feel that this difference is partially responsible for the longer action of pentobarbital. (27) Both are concentrated to approximately the same extent in skeletal muscle,(28) and skeletal muscle takes up less barbiturates than other tissues, such as liver, kidney, and brain.

The exact cellular site of action of the barbiturates has been

under investigation for many years. Most of the work has been based on the original in vitro investigations of Quastel, who, using guinea pigs, demonstrated that barbiturates inhibit respiration of brain slices and other tissues. (29,30) Grieg (31,32) in 1946 postulated that barbiturates acted specifically on the cytochrome system, with cytochrome c reductase as the probable sensitive site. Other investigators (33) have contended that the pyruvic acid oxidase system is the most sensitive. Bain (22) in his excellent review of possible modes of barbiturate action, summarizes the data of many other investigators, which show the effects of barbiturates on glucose and pyruvate oxidation by various brain preparations. From his calculations it is seen that the doses (MD_{50}) for various barbiturates required to produce 50% inhibitions of oxidations (I_{50}) would represent from four to thirty times the concentration calculated to exist in the central nervous system during actual surgical anesthesia. These calculations were based on the known distribution of these drugs in tissues. Quastel (30) in his earlier work had considered the possibility of such a discrepancy, and had concluded in his experiments that the narcotic concentrations in vivo approximated those in vitro. However, his calculations were based on theoretical blood concentrations of the drugs, and were done without benefit of the information on tissue distribution available at present.

Some investigators feel that Bain's results may be explained by a preferential concentration of barbiturates in the brain. This has been demonstrated for only two compounds, pentobarbital, which is concentrated about 1½ times, and amobarbital (ethyl, isoamyl barbituric acid) which is concentrated 4 times in brain. (34,35) Others have

felt that a so-called "critical region may exist somewhere in the central nervous system which is particularly sensitive to the barbiturates. While there is no evidence to support this postulate, Wilkins [36] and others have shown that barbiturates exert an essentially equal effect in depressing oxygen uptake in various parts of the central nervous system.

Several observations suggest that inhibition of oxygen uptake alone cannot explain the action of barbiturates as anesthetic agents. Himwich [37] has shown that depression of functional capacity precedes depression of the cerebral metabolic rate. Others [38] have shown that if nerve conduction and oxygen consumption are measured simultaneously, nerve conduction is depressed before oxygen consumption is impaired. Buchel [39] has shown that in vitro, barbiturates depress creatine phosphate and raise inorganic phosphate levels of mammalian brain, observations which are compatible with depression of oxidation. However, in vivo, after administration of barbiturates, creatine phosphate levels in brain rise with corresponding depression of inorganic phosphate.

In 1951, Brody and Bain, using rat liver mitochondrial preparations, demonstrated that thiopental inhibits the formation of high energy phosphate bonds. This was shown by a marked depression of inorganic phosphate uptake (40%) at a level of thiopental which depressed oxygen consumption less than 10%. [40] At concentrations which inhibited oxygen uptake 50%, inorganic phosphate uptake ceased altogether. These results indicate a site of action in the process coupling oxidation and phosphorylation. It is of particular significance that the concentrations of barbiturate required to produce 50% inhibition of

formation of high energy phosphate bonds correspond closely to the concentration of barbiturates in the tissues during surgical anesthesia.

The postulation of an uncoupling mechanism as a major action of barbiturates in the cell is supported by other observations. Low concentrations of pentobarbital and phenobarbital have been observed to stimulate oxygen uptake in brain slices. (41) This effect of initial stimulation with low concentration followed by depression with higher concentrations is a common one produced by known uncoupling agents, such as 2, 4-dinitrophenol. Johnson (42) postulates that such an effect may result from the accumulation of inorganic phosphate and phosphate receptors which serve to partially regulate the oxidation rate in a cell.

Holeman and Elliott (43) have shown that several barbiturates, including pentobarbital, inhibit acetylcholine synthesis in brain slices, at a concentration which produces nearly complete inhibition of inorganic phosphate uptake. Since high energy bonds are required for the synthesis of acetylcholine, these observations support the theory of an uncoupling action of these compounds.

More precise localization of the mode of uncoupling action has not yet been accomplished. Maxwell and Nickel (44) have shown, however, that the ATPase activity of rat liver mitochondria is markedly increased by 2, 4-dinitrophenol, a familiar uncoupling agent, as well as by various barbiturates. Under comparable conditions, ATPase activity in rat brain remains unaffected by these agents. When ATPase activity of rat brain is depressed by ethylenediaminetetraacetic acid, barbiturates and 2,4-dinitrophenol increase activity to a near nor-

mal level. The significance of these studies is not altogether clear, but it appears that the enzyme systems of different tissues do not necessarily respond in the same manner to barbiturates. It has been shown [45] that the ATPase activity of skeletal muscle mitochondria is also increased by uncoupling agents.

In summary, it appears at present that barbiturates have three effects upon the metabolism of cells:

(1) an inhibition of oxidation, an effect which is not marked at the usual anesthetic concentrations employed

(2) an inhibition of oxidative phosphorylation, which assumes considerable significance at ordinary anesthetic concentrations

(3) a possible stimulatory effect on ATPase activity, which apparently is not constant for all tissues, and which is effective on the ATPase of muscle mitochondria

In view of these findings, it is evident that levels of high energy phosphate compounds in muscles taken for analysis under sodium pentobarbital anesthesia might be expected to show some alteration from levels in unanesthetized animals. Since the ATP levels of muscle are maintained at the expense of the creatine phosphate reservoir, depression of the creatine phosphate fraction of muscle would not be surprising under these circumstances. One might also expect muscles subjected to the effect of the barbiturates for a longer period of time to show greater alteration. On the other hand, concentrations of pentobarbital in muscle tissue may be insufficient to exert such effects. If the increase in ATPase activity described by Maxwell and Nickel is significantly great in muscle tissue, one might expect, in addition,

a depression of ATP levels.

2. The Action of Barbiturates on Muscle Tissue

In 1955 Kreatz et al. (46) demonstrated that thiopental and pentobarbital caused potentiation of muscle contractions in innervated and denervated dog muscle. In this and additional work, they have shown that barbiturates which are most active in potentiating contraction are also those which exert the most depression on the central nervous system, and exhibit the greatest binding by tissue proteins. The most active compounds also possess a 1-methyl, butyl group. (47) These workers feel that the twitch potentiation may be attributed to depression of the "buffering" action of the "series elastic" component of muscle. Hill, in 1949, described this component as the group of elements consisting of areolar and reticular tissues between fibers, epi- and perimysium, and sarcolemma. (48) The presumed action of this component is the partial "buffering" of the muscle contraction. Depression of this element would allow a greater fraction of the true contraction to be transmitted to the recording system.

These workers also found two main physiological effects of the barbiturates on muscle contractions:

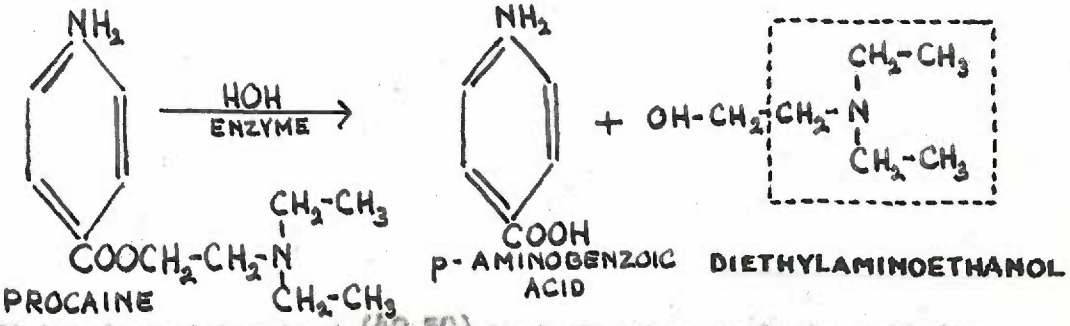
(1) Thiobarbiturates exhibit a non-specific blocking effect to administered drugs which act at the motor end-plate, such as acetylcholine, curare, and prostigmine. There is no evidence that the thiobarbiturates interfere with normal transmission at this site.

(2) The oxybarbiturates do not act in this manner, but are notable for their antagonism to transmission at the neuro-muscular junction.

B. Procaine Hydrochloride and Other Local Anesthetic Agents

1. Chemistry and Mode of Action

Procaine is the diethylaminoethyl ester of para-aminobenzoic acid. When administered by injection, it is an effective local anesthetic agent. It is rapidly hydrolyzed in the tissues to form para-aminobenzoic acid and diethylaminoethanol.



It has been determined (49,50) that the pharmacologic activity of the compound resides in the trialkylamino group of diethylaminoethanol, as shown above. A slightly alkaline medium is necessary for the hydrolysis of procaine, and normal tissues are within the effective range for this activity. (51)

As in the case of the barbiturates, the exact mechanism by which local anesthetics produce their effect on the tissue they contact has not yet been determined. Throughout the years many theories have evolved concerning this action. In some cases, many of the same mechanisms discussed earlier as possible modes of action for the barbiturates have been postulated.

Greene (52) in 1955, published an excellent review on the possible mechanisms of action of procaine and other local anesthetics. The action of a local anesthetic depends on two basic properties of nerve fibers, which are excitability and transmission ability. These two properties are dependent upon at least three mechanisms: (1) oxy-

gen consumption, (2) depolarization and repolarization, and (3) alteration in membrane permeability.

The initiation and transmission of an impulse along a nerve is associated with alterations of the membrane potential, which under resting conditions amounts to about 60-100 millivolts. The changes in membrane potential result in depolarization of the polarized adjacent membrane, and cause a wave of depolarization to spread along the nerve fiber. The membrane potential is normally dependent on oxidation for maintenance. (52) Alterations in membrane potential are also accompanied by changes in membrane permeability. Under normal conditions, the membrane of a resting nerve is more permeable to potassium ions than to sodium ions. During depolarization, a migration of sodium ions across the membrane into the cell occurs, and potassium moves across the membrane in the opposite direction, out of the cell. During the refractory period, a gradual movement of ions back through the cell membrane occurs with restoration of polarity. The active state is thus associated with a great increase in permeability of the membrane to sodium ions.

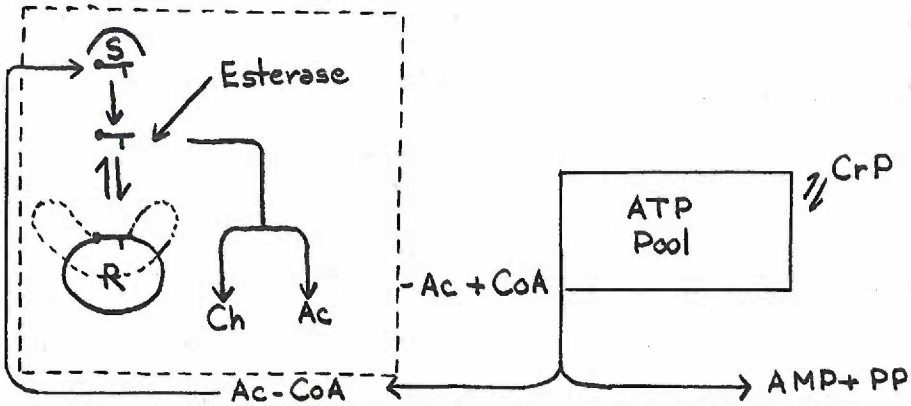
It has been shown (52) that nerve impulse transmission is associated with an increase of oxidative metabolism. However, as yet, there is no proven relation between the rate of oxygen uptake and the actual capacity for nerve tissue function. (53)

Many workers have felt that the action of procaine is due to depression of oxygen consumption in the nerve cell. While, as was pointed out earlier, all investigators agree that some anesthetics (the barbiturates) consistently depress oxygen consumption, there is less agreement

among investigators that all local anesthetics produce this effect. Many substances are capable of blocking nerve conduction. For example, potassium chloride in sufficient concentrations blocks conduction, and this is accompanied by an increased rate of oxygen uptake. On the other hand, sodium azide and hydroxylamine depress oxygen consumption without impairing conduction. Azide is known to act as an uncoupling agent, preventing oxidative phosphorylation. (54,38) Also, at concentrations which effectively prevent nerve impulse transmission, the local anesthetic, chloretone, decreases oxygen consumption, but cocaine does not. Investigators have shown that procaine, at certain concentrations, does depress oxygen uptake. (55) However, at other concentrations, it stimulates oxygen consumption. (56) In view of the more recent theories concerning the modes of action of anesthetics, it would be unreasonable to conclude that this effect alone need be the sole action of procaine, or indeed, that it is a significant action at all.

Another hypothesis concerns the effect of local anesthetics on depolarization of nerve fibers. It is of interest that high concentrations of potassium chloride, mentioned above, appear to block nerve conduction by prevention of depolarization. (57) Nachmansohn, (58) in his investigations on the action of acetylcholine at the myoneural junction, has postulated an interesting site of action for procaine and other tertiary nitrogen compounds. He believes that transmission occurs in accordance with the following scheme, in which acetylcholine is bound to a "storage protein" (S) and is released by excitatory stimuli to combine with the "receptor substance" (R). At this point the receptor changes its configuration to correspond with

the dotted line as shown below, and presumably increases the membrane permeability to sodium ions. Acetylcholine esterase (E) acts on the free ester, which is in equilibrium with the ester-receptor complex. Hydrolysis of this complex follows, and the receptor returns to its normal shape as polarity returns.



In his studies with procaine and other tertiary nitrogen compounds, Machmanschn found evidence that these substances appeared to compete with acetylcholine for the receptor substance, and probably exert their actions by preventing depolarization.

There is much disagreement among other workers regarding this possible depolarizing action of procaine. Toman (59) and others (60) have pointed out that at the usual clinical concentrations employed, procaine and cocaine do not influence membrane permeability appreciably. Also, depolarization is not produced by anesthetic doses of chloretone. Many workers feel that depolarization interference, like depression of oxygen consumption, cannot fully explain the anesthetic properties of procaine and other agents.

An older theory of procaine action postulates alteration in the permeability of the cell membrane. This was first suggested by Meyer in 1907, and later advanced by Lillie (61, 62) and Hinterstein. (63)

A corollary of this hypothesis would designate the cell surface as the primary site of action, rather than the interior of the cell. Lillie and Winterstein have demonstrated that various anesthetics, including procaine, decrease membrane permeability. Greene (52) believes that local anesthetics produce their primary effects by producing changes in membrane permeability which in turn alter the usual ionic shifts observed during nerve impulse transmission. He and others (59) believe that the mechanism of this action is by polar association of the amino group of the local anesthetic agent with polar groups in the lipo-protein of the nerve membrane.

There is also considerable disagreement among authors with regard to the applicability of the Meyer-Overton law to local anesthetics. This law states that the anesthetic activity of a chemical is related to its relative solubility in fat as compared to water, and is of importance because of the high lipid content of the central and peripheral nervous systems. Greene believes that local anesthetics follow this law. Lofgren (59) in 1948 carried out a complete physicochemical investigation of procaine, and concluded that local anesthetics do not conform to the Meyer- verton law. He believes that indifferent narcotics which adhere to the Meyer-Overton rule probably act through Vanderwaal forces, attractive forces between solute and solvent molecules. (64) In contradistinction, the true local anesthetics act through chemical combination.

Chanes (56) has contributed another pertinent observation relative to procaine action on crab nerve. He showed that with low concentrations of procaine, up to .02%, the respiratory rate was increased

to 20% above the original level. At higher concentrations, respiration was reduced, until at .3% it was only one-half of the original value. (See Figure III) This phenomenon is highly suggestive of an uncoupling action of procaine, similar to that discussed in the section on barbiturate action. At present, no investigation has been done to support this possibility.

In summary, then, the site of action of procaine and other local anesthetics is not yet clear. Many hypotheses exist, among them the depression of oxygen uptake, alteration of membrane permeability, and interference with or prevention of depolarization. The possibility of an uncoupling action remains to be considered. From the evidence at hand, it appears likely that different local anesthetics may exert entirely different effects on nerve cells to produce anesthesia.

2. The Action of Local Anesthetics on Muscle

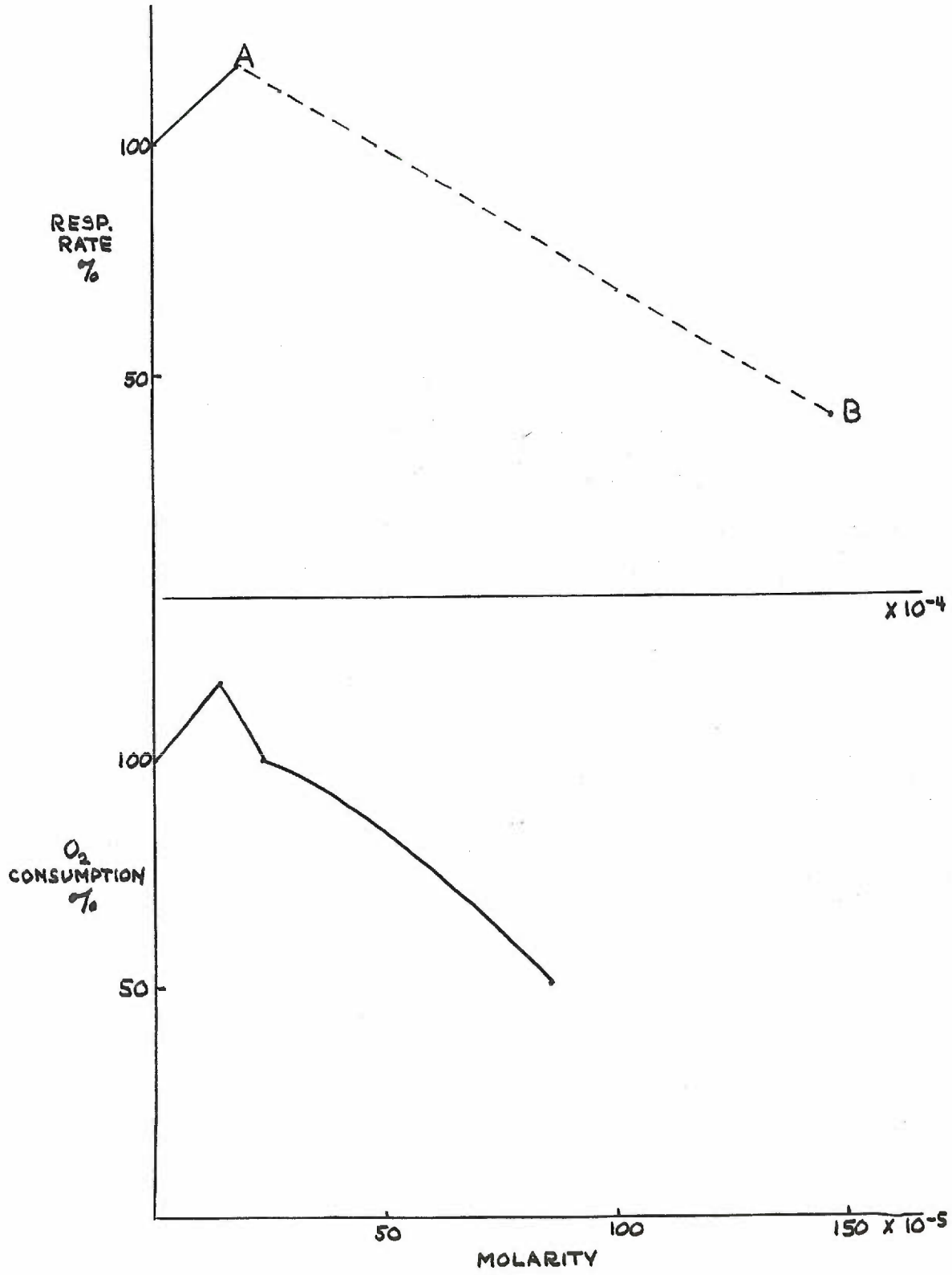
In 1919, Kubota and Macht (65) working with frog and rat Gastrocnemius, showed that immersing an excised muscle preparation in 1% procaine solution decreased muscle excitability and decreased the height of muscle contractions. Harvey (66) in 1939, investigated the effects of intra-arterial procaine injection on the Tibialis Anticus muscle of cats. He found that under such conditions the response of the muscle to direct stimulation remained normal, although response to indirect stimulation through the nerve was markedly impaired. Pace and Wood (67) were impressed with this curare-like property of procaine. Curare prevents the response of muscle to acetylcholine at the motor end-plate, although there is no inhibition of acetylcholine

FIGURE III. A Comparison of the Stimulation and Depression of Oxygen Uptake Produced by Procaine and Thiopental

1. (Above) The course of respiration in crab nerve with procaine. Dotted line represents the probable course between points A and B.

2. (Below) The oxygen consumption of rat liver mitochondria with thiopental.

FIGURE III


A
100
RESP. RATE %
50
B
X 10⁻⁴
100
O₂ CONSUMPTION %
50
50
100
150 X 10⁻⁵
MOLARITY

production. The muscle whose nerve supply has been treated with curare will continue to respond to direct chemical and electrical stimulation, but indirect stimulation does not produce contractions. Curare is directly antagonized by prostigmine and neostigmine, compounds which inhibit choline-esterase at the myo-neural junction and prolong and intensify local depolarization at the motor end-plate. Nevin (68) and others have noted that procaine infiltration in muscles of persons afflicted with myasthenia gravis leads to a great increase in muscle weakness. This disease appears to be caused by inability to utilize acetylcholine at the myo-neural junction, and is markedly improved by the compounds which inhibit choline-esterase. Isac and Wood found that procaine greatly reduces contractions after prostigmine administration. They interpreted this discovery to mean that procaine may decrease acetylcholine production, thus limiting the site of action of prostigmine. These observations are equally compatible with the hypothesis of Nachmansohn; the same effects would be seen if procaine combines preferentially with the acetylcholine receptor. However, the observation by these workers that procaine does not impair muscle contraction produced by indirect stimulation unless prostigmine is present is incompatible with previous observations.

Hellman and Katable (69) using intra-arterial injections and direct muscle injections, demonstrated that procaine depresses excitability of skeletal muscle to about the same level as that of the nerves. They feel that local anesthetics produce a general " protoplasmic depression " which affects not only nerves, but muscle and other tissues. The quantity of procaine used in these experiments appears excessive;

in addition, the fact that procaine decreases muscle excitability when administered intra-arterially or intramuscularly is hardly adequate evidence that it exerts an independent action on muscle tissue. An anesthetic administered in this manner may produce its effect through the nerves in muscle. Procaine, when administered by the usual techniques to provide local anesthesia, does not ordinarily gain such access to muscle as was provided in this experiment.

There is no indication at present that procaine hydrochloride, used as an anesthetic in local infiltration, should affect the high energy components of muscle. However, it must be borne in mind that the exact mode of action is still undetermined. Procaine, due to its rapid hydrolysis following injection, probably does not penetrate adjacent tissues appreciably. The possible effects of tissue dilution should not be overlooked when employing a local anesthetic. This factor is dependent on the technique of administration. It is obvious that any fluid injected directly into muscle tissue will alter the levels of chemical constituents of that tissue. In order to insure a realistic chemical picture of muscle tissue, care must be taken to restrict procaine injection to areas which will not be used for the determination of these elements.

III. Practical Application of Muscle Biopsy and Analysis in Research

A. Historical Background

Diseases which involve muscle, directly or indirectly, are among the many in which pathogenesis is poorly understood. Early efforts to discover possible metabolic lesions led to the recognition of the potentialities of chemical analysis of muscle. In 1934, Reinhold

et al. (70) utilized muscle analysis in an attempt to evaluate the effect of glycine administration in muscular dystrophy. Specimens of muscle were taken from the Vastus Externus, which was selected due to the frequency of its involvement in the disease. Nitrous oxide and procaine anesthesia were used, with a standard biopsy technique. The muscle was not frozen prior to analysis. Nevin (71) also in 1934, published a study of muscle chemistry in myasthenia gravis and other diseases affecting muscle. Amytal and ether were used in different cases, and muscle selection included nearly every available muscle which was encountered during various surgical procedures. In early studies such as these, very few of the factors which are considered of importance today were recognized. The importance of preservation of the labile high energy compounds by adequate freezing has now been established. Work carried out in this laboratory in the past six years has posed several additional problems in the interpretation and comparison of data.

It has been demonstrated (72) that the creatine phosphate levels in the leg muscles of rats can be decreased by adrenalectomy, and that the level can be restored by the administration of glycocyamine (guanidoacetic acid.) Rats were anesthetized with Nembutal, and the muscle biopsies obtained by a standard technique of freezing the leg, in situ, with a mixture of dry ice-acetone mush. In 1952, Dixon, Peterson, et al. (73) studied creatine phosphate levels in the anxiety fatigue syndrome by muscle biopsy and analysis, and demonstrated a significant depression of creatine phosphate levels in afflicted individuals. Clinical improvement resulted from the administration of

glycocyamine in these sub acts. In these cases, muscle biopsies were taken from controls and afflicted individuals, using thiopental anesthesia in the majority of cases. Procaine and pontocaine were used in some instances. Rectus Abdominis muscle was taken for biopsy and the standard surgical biopsy technique with immediate freezing of the muscle samples was carried out. Work in progress [74] on the effects of glycocyamine on creatine phosphate levels of muscle in multiple sclerosis, rheumatoid arthritis, and other conditions has introduced further variations into biopsy studies. Seconal and other sedatives have been used as pre-medication for the biopsy, and xylocaine hydrochloride and procaine hydrochloride, administered by local infiltration, are now the anesthetics of choice. In the work now underway, Quadriceps Femoris is generally used as the muscle for sampling, with occasional samples of Biceps and Triceps included. The standard surgical biopsy technique with immediate freezing of the sample is now in use.

B. Objectives of This Study

In the studies undertaken, involving biopsy sampling for chemical analysis, the variety of anesthetic agents used has not been eliminated as a possible important factor in results obtained. The most popular methods of anesthesia at present are the induction of general anesthesia with thiopental and the local infiltration with procaine or a related anesthetic. In some instances where thiopental is used, the time between induction and obtaining the biopsy is variable. In this project, a study of the possible variations introduced by the barbiturates and procaine is undertaken. In addition,

the possible effects of prolonged duration of anesthesia were investigated. Sodium pentobarbital (Nembutal) was used in place of thiopental because of the expense and difficulties in administration attendant to the use of the latter.

As was discussed previously, the possible sources of error derived from indiscriminate muscle selection are also of importance. A comparison of various skeletal muscles was done to establish the importance of this factor.

There is little doubt that the technique of muscle biopsy and proper disposition of the sample have an important role in the preservation of high energy compounds. Two techniques were compared in this project: the standard surgical technique which has been used in recent investigations in this laboratory, and the in situ technique, which was used in earlier investigations on the legs of rats and is still in use in this laboratory.

METHODS

A. Selection of Animals

Thirteen dogs, weighing between nineteen and twenty-five pounds, were obtained from the University of Oregon Medical School kennels for experiments performed in this project. All animals were of similar nutritional status, and efforts were made to obtain animals of tractable temperaments. Whenever possible, short-haired animals were utilized in order to expedite surgical techniques. Each animal was sacrificed following completion of the experiment in which it was used.

B. Preparation of Animals

Animals were selected several days prior to the contemplated experiment. Each animal was brought down from the kennel to the laboratory for one or more short periods, one-half to one hour, of "orientation" so that he might become accustomed to his environment, and to minimize apprehension. In several cases the animal was introduced to the dog surgery board during his initial visits.

Prior to each series of biopsies, the animal was immobilized on the dog surgery board and the site of the initial biopsy was clipped and shaved free of hair. All animals were anesthetized with sodium pentobarbital (Nembutal) administered in an initial dose of 5 milligrams per kilogram into the jugular vein. This was supplemented as necessary throughout the course of the experiment by additional intravenous injections to maintain an adequate surgical level of anesthesia. Criteria used to determine this level included the respiratory rate, heart rate, corneal reflex, complete lack of response to painful stimuli, and degree of muscle relaxation. In this exper-

iment it is particularly important to achieve an adequate degree of relaxation. In some cases, the first biopsy was taken after local infiltration with procaine hydrochloride; this was followed by immediate administration of Nembutal.

C. Biopsy Techniques

Each experiment consisted of serial muscle biopsies, selected from different muscles. At no time was a second biopsy taken from an area previously traumatized. Three biopsy techniques were employed. They were (1) the "snatch" surgical biopsy, in which the muscle was dissected free of adjacent tissue and quickly frozen; (2) the procaine biopsy, in which prior local infiltration of adjacent skin and muscle tissue with procaine hydrochloride was done; and (3) the " in situ " technique, in which the muscle was exposed, frozen in situ, and later removed. The first two techniques are comparable to two commonly used surgical techniques which have been employed in obtaining human muscle tissue analyzed in this laboratory. The third technique has been utilized on rats in this laboratory in experiments which have previously been described in this thesis.

1. The surgical "snatch" biopsy

In all cases the animal was anesthetized with sodium pentobarbital or procaine. An effort was made to approximate the same technique which is ordinarily used in obtaining human muscle biopsies, with the exception of aseptic technique. The skin over the biopsy site was shaven nearly free of hair. When suitable anesthesia was

obtained, a 4-6 cm. skin incision was made, in the same plane as the muscle fibers to be used for biopsy. Skin flaps were mobilized, and any frank bleeding was controlled with hemostats. Subcutaneous tissue and fat were dissected free of the underlying muscle. When a sufficient area (approximately 4-5 cm^2) of the underlying muscle had been exposed, a clamp was applied to a small bite of muscle tissue. The clamp was held up, with some tension exerted upon it, to facilitate removal of the specimen. Using either a scalpel or scissors, a square of muscle measuring about 3 X 2 X 2 cm. was cut free from the underlying muscle and plunged immediately, with the clamp still in place, into a mixture of dry ice-acetone "mush". The muscle sample was packed in this mixture until frozen solidly (4 minutes). The clamp was then removed and the sample was quickly wrapped in two layers of chilled aluminum foil. The samples were stored in dry ice (-70° C.). Storage at this temperature has been shown to prevent deterioration of the labile phosphate fractions for periods up to two weeks.

The biopsy technique was standardized so that all samples were taken quickly and frozen immediately. The procedure was timed from the moment of the first muscle incision to the immersion of the sample in the dry ice-acetone mixture. At no time did this interval exceed 12 seconds; in most cases it was 5-9 seconds. Immediately following the biopsy, bleeding from the biopsy site was controlled with hemostats and vessel ligation. In some instances, anti-haemophilic globulin, a convenient clotting agent, was sprinkled on the biopsy site. Wounds were then packed or sutured to prevent further bleed-

ing.

2. The "Procaine" Biopsy

In seven cases, samples of Quadriceps muscle were obtained under local procaine infiltration prior to general anesthesia with Nembutal. One per cent procaine hydrochloride with 1 ml. of 1: 10 000 epinephrine hydrochloride added per 100 ml. was used. In general, the amount used for production of the field block was 10-20 ml., and an additional 4-10 ml. was injected into the muscle adjacent to the biopsy site. This amount is roughly comparable to that used in local infiltration for human biopsies.

The field block was accomplished by subcutaneous infiltration of procaine along an imaginary square with an area of 5-7 cm^2. This technique should cause no harmful effects to the muscle tissue situated below. After waiting 1-3 minutes for local anesthetic action to become effective, a skin incision was made in the manner previously described. After dissection of subcutaneous fat and exposure of the muscle, an additional 4-10 ml. of procaine was injected deep into the peripheral muscle tissue. This additional procaine is not always used in human biopsy work. It was necessary in these cases because of the behavior of the animals, to insure adequate anesthesia and to prevent movement. Care was taken to direct the needle out and away from the part of the muscle to be taken for biopsy. It was hoped by this technique to avoid any tissue dilution of the specimen. Following this procedure, muscle was taken in a manner identical to that previously described.

3. The "in situ" technique

In nine cases, the two final biopsies taken under general anesthesia before sacrifice of the animals were of the Gastrocnemius muscles. The final sample, the Gastrocnemius on the opposite side, was taken in the following manner. The incision was made in the usual way, and the muscle exposed. In addition, the skin flaps and limiting subcutaneous tissues were drawn back in order to expose as much of the muscle as possible. A tray of dry ice-acetone "mush" and a beaker full of the mixture were prepared. The tray was placed under the muscle, and simultaneously the beaker full of the mixture was emptied over the muscle and immediately weighted down. In this way, the entire area of exposed muscle was surrounded by the dry ice-acetone mixture. Ten minutes were allowed for adequate freezing, and the frozen muscle sample was quickly dissected out with chilled bone shears, and preserved in the usual manner in cooled aluminum foil for dry-ice storage.

D. Selection of Muscles

An effort was made to secure specimens of different muscles during these experiments. An arbitrary distinction was made between the "active" muscles, which are ordinarily concerned with short bursts of tetanic contraction, and the "holding" muscles, which are concerned in postural maintenance. Thirty-seven "active" muscle biopsies were obtained under Nembutal anesthesia alone, and these included fifteen Quadriceps Femoris, two Flexor Digitorio, eleven Gastrocnemius, and nine Triceps. The "holding" muscles obtained were fifteen Rectus Abdominis, three External Abdominal Oblique, and one Internal Abdominal Oblique. In most cases, both "holding" and "active" muscles were obtained from each animal. Control samples were obtained for comparison of the "in

situ" technique, using the Gastrocnemius muscle, and the "procaine" technique, using the Quadriceps Femoris. In six instances, paired Triceps samples were obtained in which one sample of each pair was taken with local procaine infiltration, after general anesthesia with Nembutal had been accomplished.

E. Duration of Anesthesia

In order to determine the possible effects of extended duration of anesthesia, samples were taken at varying intervals up to six hours following induction. Anesthesia with Nembutal was maintained during these periods. Intervals varied from 45 to 100 minutes between samples.

F. Scheme of Analysis

Muscle samples were analyzed for inorganic phosphate, creatine phosphate, seven minute hydrolyzable phosphate fraction (which includes ADP and ATP), total acid soluble phosphate fraction, total phosphorus, dry weight, creatine, and nitrogen. In some cases analyses of the non-collagenous protein fraction were carried out.

I. Preparation of Filtrate

Round-bottomed 15 ml. centrifuge tubes, containing 2 ml. of 10% trichloroacetic acid (TCA), and fitted with rubber stoppers, were packed in dry ice for ten minutes. The tubes were wiped free of excess moisture, weighed quickly, and repacked in dry ice. Tissues were sliced with an apparatus illustrated in Plate I. It consisted of a metal plate fitted with a movable piece of angled aluminum placed on a container partially filled with dry ice. Weighed tubes were inserted through the hole in the plate to rest on the dry ice.

PLATE I. This illustrates the technique of slicing the frozen muscle (100-200 mg. samples). The muscle is held in the angled aluminum through the hole with the left hand; slices are cut with a sharp blade into the frozen tube which rests below. The angled aluminum is kept cold by dry ice resting on it.

The muscle sample was held through the opening in the angled aluminum and samples weighing 100-200 mg. were sliced with a razor blade directly into the mouth of the tube. Since brief contact with air caused condensation of moisture on the tissue slices, samples were sliced quickly. Any adherent pieces of tissue were wiped from the mouth and sides of the tube with filter paper, the stoppers were then replaced, and the tubes repacked in dry ice for a few minutes to insure adequate freezing. Tubes were quickly reweighed and replaced in the dry ice.

As each sample was sliced, one or two slices of tissue were placed on a slide, stained with Sudan III, and examined under the dissecting microscope for estimation of fat content. By an arbitrary method of fat grading in use in this laboratory, samples were classified as 0- 1+, 1+- 2+, and 2+- 3+ for fat.

The weighed frozen tissue was triturated with a footed glass rod after moistening the tissue with 3 drops of cold 10% TCA (Plate II). When the tissue had been partially broken up in a uniform manner and appeared wet, it was worked up the side of the tube with the rod, and then refrozen in dry ice. A second trituration was done to pulverize the frozen tissue. The glass rod was rinsed with 7.8 ml. of 10% TCA, making a total volume of 10 ml. (Plate III). The rubber stoppers were replaced in the tubes and the tissue homogenates frozen and thawed three times with intermittent shaking. About ten minutes was allowed to complete this process. The tubes were placed in an angle centrifuge and spun down at 1800-2000 RPM for 2 to 3 minutes. The

PLATE II. After the frozen sliced muscle in the tube has been weighed, 3 drops of 10% TCA are added. The tissue is then crushed with the footed glass rod. The original 2 ml. of 10% TCA are frozen in the bottom of the tube, with the muscle tissue resting on top.

PLATE III. Following trituration, 7.5 ml. of cold 10% TCA are added to the minced muscle for extraction of the muscle phosphates. This tube will be frozen and thawed three times to assure disruption of the cells.

supernatant fluid was then filtered through Whatman # 42 filter paper into tubes resting on dry ice. The filtrate was used for the determination of inorganic phosphate, creatine phosphate, ADP and ATP, total creatine, and the total acid soluble phosphate fraction.

II. Chemical Determinations

A. Inorganic Phosphate

Precipitation of the inorganic phosphate was carried out according to the method described by Fiske and Subbarow. (3)

Reagents:

(1) Calcium chloride reagent. 10% Calcium chloride (13.3 gm. $CaCl_2 \cdot 2H_2O$ in 100 ml. distilled water) saturated with calcium hydroxide and allowed to stand overnight.

(2) 20 % NaOH. 5 gm. NaOH in 25 ml. distilled water. Made fresh every two weeks and stored in a paraffin bottle to prevent silicate contamination from the glass.

(3) 10% trichloroacetic acid (TCA)

(4) Phenolphthalein indicator. 1 gm. phenolphthalein in 100 ml. of 95% alcohol.

Method: Three ml. aliquots of each sample of filtrate, pipetted immediately upon thawing of the frozen filtrate, were placed in 15 ml. graduated centrifuge tubes, and 20% NaOH was added from a capillary pipette until the filtrate was alkaline to phenolphthalein. One drop of indicator was added to each tube. The solution was then back-titrated with 10% TCA until a light pink color was obtained. A blank containing 3 ml. of 10% TCA was treated in the same manner as the filtrate. After neutralization, 1 ml. of 10% calcium chloride

reagent was added and the sides of the tube were rubbed with a glass rod to initiate precipitation of the inorganic phosphate fraction. The creatine phosphate remains in solution under these conditions. The calcium salt of ATP, which is formed under the conditions of this determination, begins to deteriorate after ten minutes, liberating inorganic phosphate. For this reason the samples were allowed to stand exactly ten minutes; the glass rods were then rinsed down with distilled water and removed. Tubes were centrifuged at 3000 RPM for 4 minutes, decanted, and the precipitate washed with 4 ml. of distilled water and 1 ml. of the calcium chloride reagent. Samples were then recentrifuged and drained a second time. The inorganic phosphate precipitate which remained was dissolved in 2 ml. of 10% TCA, distilled water was added to make to 10 ml., and the phosphate content was determined by the Gomori method to be described.

B. Creatine Phosphate

Two ml. aliquots of the filtrates were pipetted into 15 ml. graduated centrifuge tubes, made up to 10 ml. with distilled water, and the phosphorus content determined. Two ml. blanks of 10% TCA were treated similarly. The difference between the inorganic phosphate precipitated by calcium chloride and the phosphorus found in the TCA filtrate was taken as the creatine phosphate phosphorus.

C. ADP and ATP

The adenosine diphosphate plus adenosine triphosphate fraction of the TCA filtrate was found by determining the phosphate present after a seven minute acid hydrolysis (100 C in 1 N HCl) and then

subtracting from this value the amount of phosphate in the TCA filtrate. One ml. aliquots of the samples and 1 ml. of 10% TCA as a blank were pipetted into 15 ml. graduated centrifuge tubes, 0.2 ml. of concentrated HCl added to each, and the volumes made up to 2.5 ml. with distilled water. The tubes were covered with marbles, placed in a boiling water bath for seven minutes, and cooled in running tap water. The volume was adjusted to 10 ml. with distilled water and the phosphate content determined.

D. Determination of Phosphate

Phosphate was determined according to the method of Gomori. (75)

Reagents:

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

(2) Reducing solution. 1 gm. Elon (p-methylaminophenol sulphate) in 100 ml. of a 5% solution of sodium bisulfite.

Method: 2.5 ml. of molybdic-sulfuric acid reagent and 1 ml. of reducing solution were added to each tube containing 10 ml. of solution from the three procedures above. The volumes were made up to 15 ml. with distilled water, and the tubes fitted with tight rubber stoppers and mixed thoroughly by inverting them. Maximum optical density of the solution was reached in 45 minutes and remained stable for an additional 45 minutes. The solutions were transferred to 19 mm. cuvettes and read in the Coleman Junior Spectrophotometer at 690 mu. Each set of samples was read with its respective blank set

at 100% transmission. The mg. of phosphorus was determined from a calibration curve made with KH_2PO_4. Complete adherence to Beer's law was found in concentrations of 0.005 to 0.08 mg. of phosphorus.

Calculation: $$\text{mg. P/100 gm. tissue} = \frac{\text{mg. P (sample)} \times 100 \times 10}{\text{Tissue wt.} \times \text{ml. filtrate}}$$

E. Determination of Total Acid Soluble Phosphate

The total acid soluble phosphate represents the inorganic phosphate, creatine phosphate, ATP and ADP, and hexose phosphates. It was determined by the following technique:

One ml. of the TCA filtrate was pipetted into a test tube, and 0.25 ml. concentrated sulfuric acid and Hengar granule were added. Tubes were heated on the Kjeldahl rack until the solution became slightly brown, with escape of white fumes. Four drops of 3% hydrogen peroxide were added and samples boiled for 30 minutes. Tubes were cooled, approximately 5 ml. distilled water added, and boiled for five additional minutes to clear the solution. Samples were then cooled, diluted to approximately 20 ml. in graduated cylinders, and 5 ml. of sodium molybdate reagent and 2 ml. of Elon added to each. The solutions were diluted to 30 ml., well mixed, and allowed to stand for 45 minutes. Color development was read at 690 mu. Each sample was run in duplicate with a control sample.

Calculation: $$\text{mg. P} = \frac{\text{reading} \times 100 \times 2}{12.8 \times \text{tissue wt.}}$$

F. Determination of Creatine

Reagents:

(1) 3, 5-dinitrobenzoic acid reagent. 25 ml. of 10% aqueous sol-

ution anhydrous $NaCO_3$ was added slowly with constant mixing to 10 gm. 3, 5-dinitrobenzoic acid. 75 ml. distilled water was added and the mixture heated to boiling to dissolve the solids.

(2) 3 N NaOH

(3) 1 N HCl

(4) 10% TCA

(5) Standard creatinine solution- 10 mg. creatinine/ ml. in 0.1 N HCl. Dilute working standards 0.04 mg./ml. and 0.01 mg./ml. in 0.1 N HCl

Creatine was determined according to the method of Langley and Evans. (76)

Method: One ml. aliquots of the TCA extract filtrate were diluted to approximately 3 ml. with distilled water and the tubes covered with aluminum foil. The tubes were autoclaved in a pressure cooker at 15-20 pounds steam pressure for 30 minutes. They were then cooled, and 6 drops of 3 N NaOH added to each. Samples were diluted to 10 ml. with distilled water, and 3 ml. of 3, 5-dinitrobenzoic acid reagent quickly added, followed by 0.4 ml. 3 N NaOH. The solutions were diluted to 15 ml. with water, mixed thoroughly, and poured into cuvette tubes. Because of the photosensitivity of this reaction, tubes were allowed to stand in a dark place for ten minutes to allow color development, and read immediately against a control and blank at 495 mu. A 1 ml. aliquot of 0.4 mg. standard creatinine was run with each set of determinations.

Calculation: $\frac{\text{reading} \times 1}{295 \text{ (factor)} \times \text{tissue wt.}} \times 1.16 = \text{mg. creatine}$

G. Determination of Dry Weight

Remaining muscle tissue was allowed to thaw quickly, and cut into small pieces under a dissecting microscope, care being taken to remove all obvious fat and gross contamination with connective tissue. This procedure must be done quickly, so that the thawed muscle sample has no opportunity to lose fluid by evaporation. Lengthy manipulation of tissue predisposes to high dry weights. These pieces were placed in pre-weighed sample bottles and put immediately into an oven maintaining a temperature of approximately 90 C. Tops were removed from the bottles during the drying period. After 24 hours or longer, samples were removed from the oven and weighed. The per cent dry weight of each sample was calculated by difference.

H. Technique for Determination of Total Phosphorus

The total phosphorus represents the acid soluble fractions of muscle phosphorus plus phospholipids and phosphoproteins.

Method: 100-200 mg. samples of dry tissue were weighed out in pre-weighed heating bottles. 2-3 ml. of distilled water and 1 ml. of concentrated sulfuric acid and a Berger granule were added. Tubes were heated on a Kjeldahl rack until the solutions became brown, with escape of white fumes. Fifteen drops of 3% hydrogen peroxide were added to clear the solution. Samples were reheated and additional hydrogen peroxide added until the solutions became clear. After no color had developed over a period of thirty minutes heating, and the solutions became turbid, the bottles were cooled and 5-7 ml. distilled water added. The samples were then boiled 5 minutes to clear the solutions, cooled, and diluted to 25 ml. After thorough mixing, a 1 ml. aliquot of each solution was pipetted into

a 15 ml. graduated centrifuge tube, diluted to 10 ml., and phosphorus determined as previously described.

Calculation: $\text{mg. P} = \frac{\text{reading} \times 100 \times 25}{12.8 \times \text{tissue wt.}}$

I. Determination of Total Nitrogen

Total nitrogen was determined by modification of the method of Rinehart, Grundahl, and West. (77)

Reagents:

(1) 0.01 N HCl

(2) 10 N NaOH

(3) Boric acid 2%

(4) Methyl red-methylene blue indicator (4 drops red to one drop blue)

Method: A 1 ml. aliquot of the solution used for the determination of total phosphorus was placed in a Kjeldahl flask with a Hengar granule and 9 ml. of distilled water. The Kjeldahl flask was attached to a vacuum distillation apparatus; the receiving flask contained 25 ml. 2% boric acid and 5 drops of methyl red-methylene blue indicator. A vacuum pump was attached and the system was evacuated to 20-30 mm. mercury. The vacuum pump was detached, and 6-7 ml. of 10 N NaOH were added slowly to the Kjeldahl flask through a stopcock without breaking the vacuum. The Kjeldahl flask was placed in a boiling water bath for 10 minutes; during this period, the liberated ammonia from the Kjeldahl flask was collected in the receiving flask, which was cooled by a continuous stream of tap water. The receiving flask was then removed, and the solution back-titrated with 0.01 N HCl to a pink end point.

Calculation: mg. N/ mg. tissue = $\frac{\text{ml. HCl} \times 14 \text{ (factor)}}{\text{tissue wt.}}$

J. Determination of Non-Collagenous Protein

The non-collagenous protein was determined according to the method of Lilienthal et al. (76)

Reagents:

(1) 0.05 N NaOH

(2) Biuret reagent of Weichselbaum: 0.3% $CuSO_4$, 0.9% Na and D tartrate, and 0.5% KI in 0.2 N NaOH

Method: The determination of non-collagenous protein was made on the TCA precipitated muscle. The precipitated tissues were washed with distilled water and the supernatant carefully removed. 0.05 N NaOH was added to make a 10% weight to volume solution. After 18-20 hours at room temperature, the NaOH extract was filtered from the insoluble collagenous protein residue. The protein content of the extract was then determined. Two ml. of the extract, 3 ml. of 0.05 N NaOH, and 5 ml. of the biuret reagent were placed in a cuvette, gently mixed, and incubated in a water bath at 30 C for thirty minutes, and read at 575 mμ in the Coleman Junior Spectrophotometer.

Calculation: gm. % protein =

$$\frac{100 \times \text{mg. protein (Calibration curve)} \times \text{Vol. NaOH extr.}}{2 \times \text{tissue weight}}$$

RESULTS AND DISCUSSION

I. Interpretation and Analysis of Data

The following determinations were performed on each sample: inorganic phosphate, creatine phosphate, ATP and ADP, seven minute hydrolyzable phosphate, total acid soluble phosphate, total phosphorus, total nitrogen, creatine, dry weight, and in some cases, non-collagenous protein. In addition, the percentage of creatine bound as creatine phosphate and the ratio of creatine phosphate to non-collagenous protein were calculated. Infiltration with fat or connective tissue increases proportionately the dry weight of tissue samples, while dilution with fluid decreases dry weight. The creatine phosphate to non-collagenous protein ratio appears to be relatively constant in normal muscle; it is principally of use in the study of diseased muscle or muscle in which an abnormal biochemical state exists.

All results were corrected, prior to analysis of data, to a dry weight of 24.70. This weight was arbitrarily determined as the mean value of the dry weights of samples from the first five experimental animals. Each group of muscles analyzed, exclusive of the samples taken under procaine anesthesia and the samples with high fat content, showed mean dry weights which correlated closely with this value.

II. Comparison of the Biopsies Obtained With Procaine Anesthesia and Nembutal Anesthesia

Two groups of muscles were studied: Triceps and Quadriceps Femoris. In each group, muscles were taken using procaine infiltration and Nembutal anesthesia. Seven Quadriceps muscles were obtained by procaine in-

filtration before general anesthesia with Nembutal was induced. Fourteen samples of Quadriceps were obtained after Nembutal anesthesia. The results are presented in Table I. No significant differences were found between the two groups. The criterion of significance in this laboratory is $P < .01$. One of the samples taken under procaine infiltration showed marked deviation from the other members of the group. In this sample, lower values were obtained for inorganic phosphate, creatine phosphate, ATP and ADP, seven minute hydrolyzable phosphate, total acid soluble phosphate, creatine, and non-collagenous protein. All of these determinations were performed on the filtrate from the wet muscle homogenate. The nitrogen, total phosphorus, and dry weight of this sample, performed on dry tissue, were within the usual limits. It is felt that this discrepancy probably reflects a partial tissue dilution with the procaine solution, which was most marked in the portion of the muscle which was used for the filtrate preparation. Evidently the remainder of the muscle sample was minimally affected.

Table II shows a comparison of the Triceps muscles. The first group consists of seven samples which were obtained with procaine infiltration from animals which had been previously anesthetized with Nembutal, and were maintained under general anesthesia during the procaine biopsies. These samples might be roughly comparable to samples taken from humans in which moderate to heavy barbiturate pre-medication had been used. Nine samples were taken under barbiturate anesthesia alone. No significant difference was found between these two

TABLE I

A COMPARISON OF QUADRICEPS FEMORIS MUSCLE WITH PROCAINE INFILTRATION ANESTHESIA AND NEMBUTAL ANESTHESIA

	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	TASP	Total P (1)
PROCAINE BIOPSIES	7	Mean	14.57	51.14	40.00	108.14	132.43	183.83
		S. E.	2.07	5.37	5.18	11.68	12.29	9.46
		Range	4-20	25-68	12-56	48-142	80-172	140-214
NEMBUTAL BIOPSIES	14	Mean	18.71	54.71	40.50	113.79	142.64	205.36
		S. E.	1.49	1.07	1.03	1.98	3.43	5.73
		Range	11-30	44-68	32-49	102-125	121-180	155-240
	P		>.10	>.10	>.10	>.10	>.10	>.05

* All values expressed as mg./100 gm. as P (wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N=6

TABLE I

A COMPARISON OF QUADRICEPS FEMORIS MUSCLE WITH PROCAINE INFILTRATION ANESTHESIA AND NEMBUTAL ANESTHESIA (CONTINUED)

	NUMBER		Nitrogen * (1)	Creatine mg.%	% Rel. Creatine	Dry Weight% (1)	NCP* (1)	CrP/NCP (1)
		Mean	3.83	249.71	61.57	23.42	13.47	3.89
PROCAINE BIOPSIES	7	S. E.	.85	30.90	2.37	.87	2.06	.22
		Range	3.03-3.56	207-447	51-69	19.7-25.9	6.4-22.0	3.09-4.71
			(2)	(2)	(2)		(3)	(3)
		Mean	3.89	289.31	50.39	24.66	13.26	4.15
NEMBUTAL BIOPSIES	14	S. E.	.14	19.80	2.05	.30	.97	.16
		Range	2.44-4.65	315-523	45-71	23.2-26.5	10.7-15.2	3.43-4.58
	P		> .10	> .10	> .10	> .05	> .10	> .10

* All values expressed as gm./100 gm. (wet weight)

All values corrected to the basis of 24.70% dry weight

(1) N = 6

(2) N = 13

(3) N = 7

TABLE IX

A COMPARISON OF TRICEPS MUSCLE OBTAINED WITH PROCAINE INFILTRATION FOLLOWING NEMBUTAL ANESTHESIA AND MUSCLE OBTAINED UNDER NEMBUTAL ANESTHESIA ALONE

	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	TASP	Total P
		Mean	14.43	52.57	37.43	105.29	134.37	192.29
PROCAINE PLUS NEMBUTAL	7	S. E.	.79	3.49	2.86	6.13	9.43	7.16
		Range	11-18	37-62	28-52	76-122	111-190	173-216
		Mean	17.36	53.11	38.00	106.56	134.89	196.78
NEMBUTAL	9	S. E.	.94	2.80	.93	2.37	.98	6.40
		Range	14-23	37-65	35-43	94-116	122-151	167-229
	P		>.10	>.10	>.10	>.10	>.10	>.10

* All values expressed in mg./100 gm. as P (wet weight)

All values corrected on the basis of 34.70% dry weight

TABLE II

A COMPARISON OF TRICEPS MUSCLE OBTAINED WITH PROCAINE INFILTRATION FOLLOWING NEMBUTAL ANESTHESIA AND MUSCLE OBTAINED UNDER NEMBUTAL ANESTHESIA ALONE (CONTINUED)

	NUMBER		Nitrogen*	Creatine mg.% (1)	% Bd. Creatine (1)	Dry Weight%	NCP* (1)	Cr/NCP (1)
		Mean	3.15	326.50	66.17	24.69	12.11	4.23
PROCAINE PLUS NEMBUTAL	9	S. E.	.10	23.60	2.15	.59	.90	.32
		Range	2.74-3.60	268-396	58-73	23.4-27.9	9.04-14.6	2.72-5.04
		Mean	5.38	339.69	66.00	24.72	13.43 (2)	4.08 (2)
NEMBUTAL	9	S. E.	.10	11.2	2.41	.69	.66	.09
		Range	2.(illegible)-3.97	294-386	50-73	22.9-26.7	11.0-16.7	3.04-5.40
	P		> .10	> .10	> .10	> .10	> .10	> .10

*Values expressed as gm./100 gm. (wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N= 6
(2) N=9

groups, and no unusual values were obtained in either the Nembutal or procaine biopsies which might suggest tissue dilution.

It appears from the above results, that the methods of anesthesia used were comparable from the standpoint of biochemical analysis. It is also readily evident that careful control of the technique of procaine administration is an important factor in securing comparable results.

III. Comparison of Samples Obtained under Nembutal Anesthesia of Different Durations

For the sake of clarity, and in order to include as many samples as possible, samples were divided into three categories: (1) those taken immediately after induction and up to 100 minutes after induction of anesthesia; (2) those taken between 100 and 200 minutes following induction of anesthesia; (3) those taken after more than 200 minutes after induction of anesthesia. Eleven muscles were included in the first group, ten muscles in the second group, and five muscles in the third group. All muscles selected for this comparison were "active" muscles. No significant differences were found between the muscles taken in the first period and those taken in the later periods. (Table III) Under the conditions of this experiment, barbiturates do not appear significantly to influence the biochemical stability of skeletal muscle over prolonged periods of administration, as measured by the techniques in this laboratory.

IV. A Comparison of the "Active" and "Holding" Muscles

The arbitrary distinction made between these two groups of muscles has been described previously. Thirty-seven active muscles were

TABLE III

A COMPARISON OF ACTIVE MUSCLES OBTAINED UNDER VARYING DURATIONS OF ANESTHESIA

MINUTES	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	THP	Total P
		Mean	17.91	54.75	41.00	113.45	142.64	305.73
Immediate-100	11	S. E.	1.73	2.51	1.19	1.64	4.19	7.32
		Range	11-30	44-68	32-49	107-126	131-160	155-246
		Mean	15.70	51.80	37.70	108.50	133.20	193.80
100-200	10	S. E.	1.25	2.49	.12	3.17	3.72	4.68
		Range	13-24	37-65	31-43	94-125	111-154	175-213
		P	>.10	>.10	>.05	>.10	>.10	>.10
		Mean	20.60	55.80	37.60	112.20	134.40	184.40
200	5	S. E.	1.43	2.65	1.03	3.97	2.68	9.13
		Range	17-2[illegible]	[illegible]-64	35-40	102-123	127-139	163-208
		P	>.10	>.10	>.05	>.10	>.10	>.10

* All values expressed as mg./100 gm. as P (wet weight)

All values corrected on the basis of 24.70% dry weight

TABLE III

A COMPARISON OF ACTIVE MUSCLES OBTAINED UNDER VARYING DURATIONS OF ANESTHESIA
(CONTINUED)

MINUTES	NUMBER		Nitrogen*	Creatine mg.%	% Av. Creatine	Dry Weight%	NCP* (1)	CrP/NCP (1
		Mean	3.32	376.90	68.80	26.69	12.83	4.21
Immediate-100	11	S. E.	.17	14.10	6.60	.32	3.46	.17
		Range	2.44-4.65 (2)	313-449	49-71	23.2-26.5	10.9-17.8 (1)	3.42-4.56 (1
		Mean	3.26	362.50	61.10	25.54	14.18	3.69
100-200	10	S. E.	.08	22.30	2.65	.79	3.40	.17
		Range	2.86-3.64	292-323	45-72	23.0-29.2	11.0-16.7	3.04-4.93
	P		>.10	>.10	>.10	>.10	>.10	>.10
		Mean	3.01	365.40	59.00	24.93		
200	5	S. E.	.13	22.60	5.93	.47		
		Range	2.38-3.42	337-444	50-84	23.9-26.5		
	P		>.10	>.10	>.10	>.10		

(1) N=6
(2) N=9

* Values expressed as gm./100 gm (wet weight)
All values corrected on the basis of 24.70% dry weight

compared with nineteen holding muscles (Table III). No significant differences were demonstrated between the inorganic phosphate, creatine phosphate, total nitrogen, creatine, per cent bound creatine, non-collagenous protein, and creatine phosphate to non-collagenous protein ratio. The levels of ATP plus ADP, seven minute hydrolyzable phosphate, total acid soluble phosphate, and total phosphorus fractions of the holding muscles were significantly lower than those of the active muscles. The average dry weight of the holding muscles was significantly higher than that of the active muscles. In examining the individual muscles comprising each series, it was noted that the holding muscles contained a much higher proportion of 2+-3+ fat samples than the active muscles. Six of the nineteen holding muscles were in this category, as compared to three of the thirty-seven active muscles. Therefore, in order to determine what variations might be due to differences in fat content, a comparison of seven samples graded 1+-2+ fat with nine samples graded 2+-3+ fat was done.

Table I shows that samples graded 2+-3+ fat differ significantly from samples graded 1+-2+ fat in ATP plus ADP, total acid soluble phosphate, and total phosphorus. In each case the 2+-3+ samples were significantly lower than the 1+-2+ samples. No explanation of these apparently random differences can be offered, but it appears evident that some of the difference between the active and holding muscles might be explained in this particular series by the higher fat content of the holding muscles.

In order to demonstrate whether or not all of this effect was

TABLE II

A COMPARISON OF ACTIVE AND HOLDING MUSCLES

	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	TAEP	Total P (1)
		Mean	17.74	52.68	30.00	100.47	125.26	167.17
HOLDING MUSCLES	19	S. E.	1.23	1.79	1.15	2.87	3.76	5.09
		Range	11-27	36-66	20-39	67-116	83-151	107-202
		Mean	10.68	53.19	36.22	110.24	136.62	194.32
ACTIVE MUSCLES	37	S. E.	.70	1.16	.64	1.29	1.92	3.59
		Range	11-30	37-83	30-49	94-135	111-160	155-246
	P		>.10	>.10	<.005	<.005	<.005	<.005

* All values expressed as mg./100 gm. as P (wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N=18

TABLE IV

A COMPARISON OF ACTIVE AND HOLDING MUSCLES (CONTINUED)

	NUMBER		Nitrogen* (1)	Creatine mg.%	% Bd. Creatine	Dry Weight%	NCP* (2)	CrP/NCP (2)
		Mean	3.11	368.00	61.42	27.21	12.98	4.20
HOLDING MUSCLES	19	S. E.	.08	11.10	.95	.69	.94	.32
		Range	2.84-3.34 (3)	322-421 (3)	60-68 (3)	24.2-37.5	9.3-18.6 (4)	3.08-5.94 (4)
		Mean	3.29	362.47	62.64	24.94	15.48	3.99
ACTIVE MUSCLES	37	S. E.	.07	8.69	1.18	.87	.47	.14
		Range	2.38-4.65	272-523	45-73	23.0-29.2	10.7-18.7	3.30-4.95
	P		>.10	>.10	>.10	<.005	>.10	>.10

* Values expressed as gm./100 gm. (wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N= 19
(2) N=9
(3) N=36
(4) N=21

TABLE V

A COMPARISON OF MUSCLES GRADED 1+- 2+ FAT

WITH MUSCLES GRADED 2+- 3+ FAT

FAT	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	TASP	Total P
		Mean	15.71	49.43	39.39	104.00	153.71	180.86
1+- 2+	7	S. E.	1.22	1.94	2.54	4.16	29.30	29.80
		Range	11-19	44-58	31-52	95-122	118-188	143-214
		Mean	15.67	47.67	28.78	92.33	118.00	159.11
2+- 3+	9	S. E.	1.29	2.90	1.91	4.00	17.00	29.70
		Range	11-20	37-58	20-37	67-105	83-136	107-208
	P		>.10	>.10	<.005	>.05	<.005	<.005

* All values expressed as mg./100 gm. as P (wet weight)

All values corrected on the basis of 24.70% dry weight

TABLE V

A COMPARISON OF MUSCLES GRADED 1+- 2+FAT

WITH MUSCLES GRADED 2+- 3+FAT (CONTINUED)

FAT	NUMBER		Nitrogen*	Creatine mg%	% Bd. Creatine	Dry Weight%	NCP* (1)	CrP/NCP (1)
		Mean	3.09	348.71	60.43	24.86	12.77	3.88
1+- 2+	7	S. E.	.09	15.80	2.48	.85	.04	.17
		Range	2.74-3.28	282-400	53-71	23.6-27.8	10.2-14.5	3.31-4.78
							(1)	(1)
		Mean	3.19	325.56	62.00	28.27	11.76	4.11
2+- 3+	9	S. E.	.10	11.40	1.81	1.42	.46	.29
		Range	2.54-3.54	222-388	50-68	23.3-37.8	9.3-14.2	3.30-5.14
	P		>.10	>.10	>.10	>.05	>.10	>.10

* Values expressed as gm./100 gm. ms(wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N = 6

TABLE VI

COMPARISON OF ACTIVE AND HOLDING MUSCLES.

EXCLUDING Sr- S+ FAT SAMPLES

	NUMBER		IP*	CrP	ATP ADP	7 min. hydrol. P	TASP	Total P
HOLDING MUSCLES	13	Mean	18.69	53.77	31.69	105.54	150.54	177.25
		S. E.	2.00	3.10	.95	2.28	3.61	3.92
		Range	11-27	42-66	27-39	92-116	108-151	143-202
ACTIVE MUSCLES	34	Mean	18.85	56.15	38.05	111.35	157.85	195.89
		S. E.	.76	1.09	.51	1.20	1.99	3.64
		Range	11-30	37-88	32-49	95-125	111-180	133-246
	P		$>.10$	$>.10$	$<.005$	$<.02>.01$	$>.05$	$<.02>.01$

* All values expressed as mg./100 gm. as P (wet tissue weight)

All values corrected on the basis of 24.70% dry weight

TABLE VI

COMPARISON OF ACTIVE AND HOLDING MUSCLES,

EXCLUDING 2+- 3+ FAT SAMPLES (CONTINUED)

	NUMBER		Nitrogen gm. % (1)	Creatine mg. %	% Bd. Creatine	Dry Weight %
		Mean	3.09	375.08	80.31	26.12
HOLDING MUSCLES	12	S. E.	.10	10.50	1.12	.34
		Range	2.37 - 3.46	324- 421	50-86	24.2- 27.9
			(2)	(2)	(2)	
		Mean	3.40	367.30	80.03	24.88
ACTIVE MUSCLES	34	S. E.	.72	8.95	1.32	.81
		Range	2.39- 4.65	272- 503	45-73	22.4- 29.2
	P		>.36	>.10	>.19	<.02>.01

All values corrected on the basis of 24.70% dry weight

(1) N = 12

(2) N = 33

due to high fat content samples, active and holding muscles were compared again, excluding the 2+- 3+fat samples from each group (Table VI). Even without the high fat content samples, a highly significant difference in the ATP plus ADP content of these two groups is evident. The holding muscles had a significantly lower ATP than the active muscles, and the differences between the seven minute hydrolyzable phosphate and total phosphorus, while reduced somewhat by the exclusion of the samples of high fat content, are still suggestive of a real difference. (P<.02 P>.01) The significance of the difference in the ATP content is, in fact, even greater in the muscles in Table VI than when the fat samples are included (Table IV). This increase in significance is explainable by the fact that the standard error of the ATP fractions is decreased by the exclusion of the high fat samples. The apparent difference between the active and holding muscles in ATP levels, without concomitant change in the creatine phosphate levels, cannot be readily explained.

V. A Comparison of Two Methods of Biopsying Gastrocnemius Muscles

The techniques used for procuring Gastrocnemius samples have been described. A comparison was made of nine muscles obtained by the "in situ" technique and eleven muscles obtained by the standard surgical biopsy technique (Table VII). The two groups appear to be comparable to each other, since no significant differences were found for any of the analyzed components. It may be concluded that adequate comparable preservation of the muscle may be achieved by either technique, so long as freezing is efficient.

TABLE VII

A COMPARISON OF STANDARD SURGICAL BIOPSY TECHNIQUE AND "IN SITU" FREEZING TECHNIQUE ON GASTROCNEMIUS MUSCLE

	NUMBER		IP*	CrP	ATP ADP	γ min. hydrol. P	TASP	Total P
IN SITU	9	Mean	19.66	51.56	35.44	106.56	138.89	184.44
		S. E.	.87	2.08	.94	3.08	4.98	6.60
		Range	16-24	43-60	31-40	91-119	110-154	148-208
SURGICAL BIOPSY	11	Mean	19.82	52.36	37.09	110.09	135.45	183.64
		S. E.	1.10	2.14	.96	3.43	1.84	5.83
		Range	13-25	41-65	30-40	96-125	121-145	163-214
	P		>.10	>.10	>.10	>.10	>.10	>.10

* All values expressed as mg./100 gm. as P (wet weight)

All values corrected on the basis of 24.70% dry weight

TABLE VII

A COMPARISON OF STANDARD SURGICAL BIOPSY TECHNIQUE

AND "IN SITU" FREEZING TECHNIQUE ON GASTROCNEMIUS MUSCLES

(CONTINUED)

	NUMBER		Nitrogen* (1)	Creatine mg%	% Bd. Creatine	Dry Weight %	NCP (2)	CrP/NCP (2)
		Mean	3.26	323.11	68.56	24.18	14.08	3.41
IN SITU	9	S. E.	.02	26.30	4.39	.23	1.29	.19
		Range	2.94-3.48	193-468	51-94	23.4-24.7	11.7-16.8	2.93-3.98
		Mean	3.23	351.36	63.73	24.66	15.80 (3)	3.69 (3)
SURGICAL BIOPSY	11	S. E.	.10	16.09	1.88	.31	.61	.27
		Range	2.36-3.64	293-444	50-72	23.5-25.6	12.4-16.3	3.09-4.93
	P		>.10	>.10	>.10	>.10	>.10	>.10

* Values expressed as gm./100 gm. (wet weight)

All values corrected on the basis of 24.70% dry weight

(1) N=8
(2) N=8
(3) N=6

SUMMARY

1. The effects of Nembutal anesthesia, procaine anesthesia, duration of anesthesia, muscle selection, and technique of biopsy on the high energy phosphate fractions in the muscles of dogs has been investigated.

2. No significant differences were found between any of the fractions analyzed in comparable muscles taken under Nembutal anesthesia and those taken with procaine infiltration.

3. No significant difference could be demonstrated between muscles biopsied soon after induction of anesthesia with Nembutal and those biopsied at varying intervals thereafter.

4. A significant difference between the active and holding muscles was demonstrated. There was a lower level of ATP, seven minute hydrolyzable phosphate, total acid soluble phosphate, and total phosphorus in holding muscles. The dry weights were significantly higher in the holding muscles. These differences may be partially explained by the differences in fat content between the two muscle groups. A significantly lower level of ATP in the holding muscles was demonstrated which could not be explained by differences in fat content.

5. Muscles graded 2+-3+ fat contained significantly lower ATP, total acid soluble phosphate, and total phosphorus fractions than muscles which were graded 1+-2+ fat. High fat content appears to be associated with unreliable results.

6. No differences were found in any of the fractions analyzed, between muscles obtained by the standard surgical biopsy and those from *in situ*.

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