MECHANISM OF ACTION OF HYPOXIA IN AIRWAY SMOOTH MUSCLE: EFFECT ON ENERGY AND CALCIUM METABOLISM

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Edwin A. Kroeger

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ABSTRACT

The present studies were undertaken to determine the role of energy metabolism, calcium content and exchange kinetics in the mechanical manifestations of the effect of hypoxia on canine tracheal smooth muscle. The effects were: 1) a shift of the force-velocity relationship to the left and 2) a spontaneous rise in resting tension at a critical combination of hypoxia and substrate depletion. The hypothesis tested was that in hypoxia with exogenous substrate depletion, endogenous substrate stores, primarily glycogen, are utilized fairly rapidly in the glycolytic pathway after which high energy phosphate stores decrease with correspondingly decreased function of the contractile elements. Measurements of glycogen, creatine phosphate (CP), adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) showed that, indeed, in hypoxia glycogen was depleted rapidly and the high energy phosphates were decreased with CP falling to 17% and ATP to 61% of control levels. ADP showed little change and AMP increased by a factor of four. The findings with reference to energy stores clearly support the hypothesis that a decrease in high energy phosphates is related to the previously observed decrease in contractile function of the muscle. The postulated decrease in energy stores would also presumably be reflected in decreased efficiency of other active processes such as the active transport of calcium ions (Ca^{++}) out of the myoplasm. The resulting accumulation of Ca^{++} intracellularly could

initiate the observed rise in resting tension. This possibility was also tested. The calcium content of hypoxic tissues equilibrated in a normal calcium solution was significantly increased while the calcium content of hypoxic tissues equilibrated in a Ca^{++}-free medium was significantly decreased as compared to appropriate controls. Water shifts were not detected. Analysis of calcium efflux curves utilizing tracer Ca^{45} revealed at least three exponential components with half times of 0.72 min, 12.22 min and 473 min, though the method did not provide adequate resolution to show a significant change of these values in hypoxia. If however a muscle was made hypoxic at a time when the rate of calcium efflux had become quite low, the efflux rate increased at the same time that the resting tension was observed to rise. The data on calcium content and kinetics are consistent with the concept of two calcium pumps: the observation that tissue calcium was decreased by hypoxia in a Ca^{++}-free environment and that Ca^{45} efflux was increased by hypoxia suggest that one of these is operative at some intracellular site while the finding of an increased tissue calcium level in hypoxia in the presence of external Ca^{++} is suggestive of a Ca^{++} pump in the sarcolemma. On inhibition of both pumps, the tissue content of calcium should reflect the extracellular calcium concentration as was observed. It is logical to suppose that the increased release of Ca^{45} from the tissue in hypoxia reflects decreased active binding of calcium in some pool with a long half time of exchange which has, thus, retained a relatively high specific activity of Ca^{45}. These results, although not conclusive in that intra-

cellular Ca^{++} concentrations were not directly measured, are consistent with the hypothesis tested.

Thus the findings indicate that the effect of hypoxia is to decrease energy stores with a concomitant decrease in contractile function, increase in net Ca^{++} influx and increase in resting tension.

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INTRODUCTION

A. GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

The effect of hypoxia on smooth muscle mechanics in general is to de- decrease spontaneous activity and active mechanical responses to stimulation (Bean and Sidkey, 1957; Detar and Bohr, 1968; Bueding and Hawkins, 1964). The unstimulated or resting tension is also usually reduced in hypoxia.

The effect of hypoxia on airway smooth muscle appears to be to contract this muscle (Tisi et. al., 1970; Widdicombe, 1966) though negative results have also been reported (Nissel, 1950). These conclusions have been reached on the basis of resistance, compliance and dead space measurements. Since these lungs were denervated and isolated to varying degrees and since the methods employed did not directly measure smooth muscle tone, it is difficult to determine whether the responses seen were the result of a direct effect on airway smooth muscle. The finding by Staub (1963) that the P_{O_2} of blood perfusing the terminal bronchiolar smooth muscle is markedly affected by the P_{O_2} of ventilatory gases suggests that hypoxia could produce local effects on the mural smooth muscle. Studies on isolated bronchial and tracheal smooth muscle (Stephens et. al., 1968; Stephens and Kroeger, 1970) have shown that the effect of hypoxia was to decrease active contractile function and to increase resting tension.

An understanding of the function of airway smooth muscle and the effect of hypoxia requires a study of the physiology of this muscle. These studies are best conducted by investigating the muscle in terms of its function and the

mechanism by which control of that function is normally effected. Since the function of muscle is twofold, 1) to stiffen and bear weight and, 2) to shorten and move a load through a distance, any quantification of muscle function must be in terms of these parameters.

Muscle physiology has been studied isometrically in terms of its length-tension (L-T) relationship, isotonically in terms of absolute shortening and by a combination of the two in which, while supporting a given load, the maximum velocity of shortening is measured. This is the force-velocity (F-V) relationship for the muscle. Since the first two of the methods mentioned yield information only about the energy utilized in activation of the muscle and in doing work, and do not measure power production or take account of the frictional losses of energy sustained in carrying out work at different velocities, they are inadequate indices of muscle function. Csapo (1954) has shown, for example, that temperature may have a marked effect on active tension development but little effect on the magnitude of isotonic shortening. Inasmuch as it allows study of the contractile element uninfluenced by the elastic properties of the series elastic component, the delineation of the force-velocity relationship of the isotonically contracting muscle is of decided advantage. The classical work of A.V. Hill (1938) on voluntary striated muscle has shown that muscle function is most meaningfully studied in terms of its force-velocity relationship. Since it provides an index of power production, the F-V relationship provides a better account of energy utilization of the working muscle than

the other methods cited.

Such an approach has been adopted by Stephens and co-workers (Stephens et. al., 1969; Stephens and Kroeger, 1970) and it has been found that the F-V relationship of trachealis muscle is shifted to the left in hypoxia.

In searching for the mechanism causing the mechanical manifestations of hypoxia various hypotheses have been put forward. Bergofsky and Weinberg (1965) in investigating the pulmonary pressor response to hypoxia have suggested that the muscle may be partially depolarized and that the resulting hyperexcitability causes the response. Lloyd's (1967) studies do not support this hypothesis, however. Detar and Bohr (1968 b) in studying the effects of hypoxia for a prolonged period in rabbit aorta have observed that this muscle becomes dependent on continued hypoxia to maintain its contractile function. While this interesting observation might appear at first glance to imply enzymic changes related to adaptation to this environment, it is well known that mitochondria are uncoupled by severe, prolonged hypoxia and that decreased pH is a potent stimulus to glycolysis. A simpler explanation of this phenomenon, therefore, is that, on the readmission of oxygen, the uncoupled mitochondria in the muscle rapidly utilize substrates such as lactic and pyruvic acids, thus increasing the pH and slowing glycolysis without liberating additional energy. On the basis of this hypothesis, however, the additional postulate that in prolonged substrate-free hypoxia the mitochondria

are reversibly uncoupled would have to be put forward to explain the oxygen dependence of contraction of substrate-free hypoxia adapted muscles.

Statement of Problem

We have previously (Stephens and Kroeger, 1970) shown that the effect of hypoxia on canine trachealis smooth muscle mechanics in vitro was to shift the force-velocity relationship to the left. This shift was potentiated by exogenous substrate (dextrose) depletion. When a combination of dextrose depletion and hypoxia had decreased mechanical function critically (about 70%) a spontaneous small rise in resting (unstimulated) tension was observed. Neither of these observations could be explained in terms of membrane phenomena such as depolarization block or hypersensitivity or in terms of spontaneous neural activity in the preparation.

Some reports (Somlyo and Somlyo, 1968) have emphasized the importance of glycolysis in energy liberation in smooth muscle. Thus a study of endogenous substrate and high energy phosphate stores was deemed essential to an understanding of the mechanical manifestations of hypoxia. The hypothesis tested was that in hypoxia glycogen and high energy phosphate stores decreased, with a concomitant decrease in active processes such as active tension development. Since Ca^{++} ions are actively transported out of the myoplasm and are important in the activation of the contractile process, experiments were also designed to detect calcium movements which could initiate the observed rise in resting tension. These included

the measurement of tissue calcium and Ca^{45} release kinetics under various experimental conditions.

B. ENERGY METABOLISM IN MUSCLE

I. Energy metabolism in striated muscle

a. Introduction

Since stimulated muscles have the capacity to shorten and do work with associated heat production, this utilization of energy by an isothermal system implies endogenous mechanisms for the liberation and storage of potential energy. That this energy is derived from chemical bond energy liberated in chemical reactions within the cell has been appreciated since the beginning of this century.

Since most of the information regarding muscle biochemistry has come from work on striated muscle it will be reviewed as it forms a basis for our understanding of smooth muscle metabolism which is reviewed in a separate section. The evolution of modern concepts in the field is reviewed below.

b. Lactic acid theory

Although the production of lactic acid in muscle contraction had been implicated in a number of earlier studies, Fletcher and Hopkins (1907) using a quick-freezing technique, were the first to obtain quantitatively reproducible data on the correlation of lactic acid production and fatigue in anaerobic muscles.

A number of findings relevant to the position of lactic acid in carbohydrate metabolism and its relation to contraction were subsequently reported by other investigators. Parnas and Wagner (1914) showed that muscle glycogen was the substrate for the formation of lactic acid. Meyerhof (1920-24) studied the problem in some depth and confirmed the observations of Parnas and Wagner for both intact and minced muscle. In addition to observing the role of phosphate in glycolysis and the resynthesis of glycogen, he measured the heat produced by muscle coincident with lactic acid production. When the values obtained from resting muscle (280-300 cal/g lactate) were corrected for the ionization heat of protein buffers, they compared very well with the free energy change involved in the conversion of glycogen to lactic acid reported by Meier and Meyerhof (1924). The discrepancy resulting from the higher value of 380 cal/g determined from tetanized muscles could, however, not be explained. Since these muscles had been observed to contract anaerobically as well as with oxygen, it is not surprising, in view of the findings of lactic acid production from endogenous stores and the favourable energetics of this transformation, that the extrapolation was made implicating lactic acid production as the cause of contraction in spite of a lack of direct supporting evidence.

c. Creatine phosphate theory

The first indication of a serious flaw in this theory came from Embden's laboratory (Embden et. al.,1926; Lehnartz, 1928; Embden et. al., 1928) in which it was demonstrated by both Embden and Lehnartz that by direct and

indirect measurements a portion of the lactate was formed after the termination of contraction. These studies were the source of some controversy and were not accepted until Lundsgaard's work (Lundsgaard, 1930 a, b, 1931, 1934) clearly showed that, by blocking glycolysis with iodoacetate (IAA) muscle contraction could be dissociated from lactic acid production and that the contractile process was not affected by the inhibition. Under these conditions no lactic acid was produced in contraction but instead creatine phosphate (CP) was observed to decrease and there was a good correlation between CP content and strength of contraction. Thus he concluded that lactic acid was not causally related to contraction. On the other hand, his observation of the correlation of CP content and contractile activity in IAA-poisoned muscles as well as Nachmansohn's (1928) observation of the resynthesis of CP immediately after relaxation in anaerobic muscles led Lundsgaard to propose (1930 a, b) that in poisoned and, most probably in normal muscle the hydrolysis of CP supplied the energy for contraction. That this compound contained enough energy to drive the contractile process had been suggested from the experiments of Meyerhof and Lohman (1927) which had indicated that the heat of hydrolysis of creatine phosphate was 12000 cal/mole.

d. The role of ATP

Lundsgaard's latter postulate, however, like the lactic acid theory, was not supported by direct evidence. Direct evidence that it was not the immediate source of energy for muscle cells was obtained by Lohmann (1934) when he added creatine phosphate to a cell-free muscle extract and observed that

this compound was not dephosphorylated in the absence of adenosine nucleotides. Since two reactions could be demonstrated to be involved in the dephosphorylation; creatine phosphate rephosphorylating the product of adenosine triphosphate (ATP) hydrolysis, it was concluded that ATP was the immediate source of energy for contraction.

While these findings and others gave additional indirect support to the notion of the central role of ATP, difficulties in demonstrating the predicted utilization of ATP in the contraction of living muscle cast some doubt on this theory some twenty-years later. We shall return to the subject of the role of high energy phosphates after summarizing the present knowledge of biochemical reactions in glycolysis and the tricarboxylic acid cycle in which the chemical bond energy of various substrates may be conserved.

e. Carbohydrate metabolism in muscle

1. Introduction

Although the specific substrates for energy metabolism in muscle appear to vary with the particular muscle under study and its state of activity (Chapler and Stainsby, 1968) the major biochemical pathways by which working muscles liberate energy are undoubtedly the glycolytic and tricarboxylic acid cycles (West et. al., 1966). Cardiac and resting skeletal muscle, however, utilize lipids to a large extent in energy metabolism. While these pathways are discussed in detail in all current textbooks of biochemistry, we shall review

some of the salient features in this context.

2. Inter-relationship of glucose and glycogen

Glucose after entering the cell, enters the metabolic pathways by being phosphorylated by hexokinase to glucose-6-phosphate, the phosphate being donated by ATP in the presence of Mg^{++}. At this point it may be utilized to synthesize glycogen through being converted by phosphoglucomutase to glucose-1-phosphate. Uridine diphosphate glucose is then formed, the enzyme uridine diphosphoglucose pyrophosphorylase catalyzing the reaction with uridine triphosphate. Glycogen synthetase effects the addition of these glucose units to the non-reducing end of glycogen by 1,4 linkages and a branching enzyme, by forming 1,6 bonds from 1,4 bonds after chains of a critical length have been formed, creates the branched structure characteristic of glycogen (Larner, 1953). This pathway for glycogen synthesis is practically irreversible.

The enzyme, glycogen synthetase occurs in two forms: a dependent (D) form whose activity is largely dependent on the presence of glucose-6-phosphate and an independent (I) form which does not exhibit this dependence. The D form is converted to the I form by the removal of phosphate and, in fact such a conversion is effected fairly rapidly on activation of the muscle (Danforth, 1965). Thus, through some form of negative feedback control the tissue glycogen level acts to control its own rate of synthesis by adjusting the ratio of synthetase I/D, but the exact nature of the interaction is complex (Larner et. al., 1964).

Glucose-6-phosphate can be reformed from glycogen by the glycogenolytic action of phosphorylase enzymes and conversion of the resulting glucose-1-phosphate to glucose-6-phosphate by phosphoglucomutase. Since phosphorylase can only cleave the straight chain 1,4 bonds, however, it acts in conjunction with amylo-1,6-glucosidase to degrade the branched glycogen structure. Phosphorylase, like synthetase, exists in two forms, with different activities. The activity of phosphorylase b is absolutely dependent on the presence of AMP, and, since the latter is found in low concentration in the cell, this is often referred to as inactive phosphorylase. The activity of phosphorylase a is little dependent on AMP. The two forms of phosphorylase are readily interconvertible, the b form, a dimer, being formed by the hydrolysis of the a form, which is a tetramer. The phosphorylation reaction is catalyzed by active phosphorylase b kinase and requires ATP in the presence of Mg^{++}. Inactive phosphorylase b kinase is activated by protein kinase which, in turn, is activated by cyclic 3'5' AMP. This is formed from ATP by adenyl cyclase. Since epinephrine, electrical stimulation and increased Ca^{++} concentrations activate adenyl cyclase, the action of almost any physiological stimulus may be expected to be reflected in muscle glycogen levels. Similarly, the effect of inhibitors of phosphodiesterase which catalyzes the degradation of cyclic 3'5' AMP is reflected in decreased glycogen levels.

In view of the fact that the phosphorylase reaction is readily reversible one might wonder as to why it should be necessary to have two different path-

ways to accomplish the same purpose. It has been shown however, (Strominger, 1960; Larner et. al., 1960) that under resting conditions where glycogen synthesis is known to occur that the equilibrium predicted from reactant concentrations is not favourable to glycogen synthesis by phosphorylase. Furthermore, increases in phosphorylase activity consistently led to the degradation of glycogen in experimental preparations rather than occasional synthesis which should have been expected had it been also catalyzing the synthesis of glycogen (Leloir, 1964).

3. Glycolysis - The Embden-Meyerhof pathway

Phosphoglucose isomerase reversibly converts glucose-6-phosphate to fructose-6-phosphate. The phosphorylation of the latter compound to fructose-1,6-diphosphate by phosphofructokinase requires ATP and Mg^{++} and is of special interest since the reaction is practically irreversible (the reverse reaction is catalyzed by fructose-1,6-diphosphatase) and is inhibited by ATP. This inhibition is relieved by AMP and inorganic phosphate. With increased energy (ATP) utilization the ATP concentration falls and the concentrations of AMP and Pi rise, thus activating phosphofructokinase and accelerating glycolysis, since the other reactions in the sequence are not rate-limiting. Thus the properties of phosphofructokinase enable it to match energy liberation with energy utilization.

The enzyme aldolase splits fructose-1,6-diphosphate centrally yielding one molecule of each of glyceraldehyde-3-phosphate and dihydroxyacetonphosphate. These are interconvertible through the action of the enzyme triose phosphate isomerase.

Glyceraldehyde-3-phosphate is then oxidized and phosphorylated by glyceraldehyde-3-phosphate dehydrogenase to 1,3-diphosphoglyceric acid. Nicotinamide adenine dinucleotide (NAD^+) is reduced in this reaction. The subsequent dephosphorylation of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid by 3-phosphoglyceric acid kinase phosphorylates ADP to ATP. 3-Phosphoglyceric acid is then converted from the low energy phosphate state through 2-phosphoglyceric acid to phosphoenol pyruvic acid. This molecule contains a high-energy phosphate group which can be transferred to ADP on oxidation to pyruvic acid by pyruvic kinase.

Although pyruvic acid is the terminal compound in glycolysis proper, its relationship to lactic acid is of particular importance to the tissue in hypoxia when mitochondrial oxidation of NADH is inhibited. Since NAD^+ is reduced in the oxidation of glyceraldehyde-3-phosphate and the supply of NAD^+ is limited, glycolysis would be rapidly inhibited if the NADH formed in this reaction were not oxidized. The formation of lactic acid from pyruvic acid by lactic acid dehydrogenase oxidizes NADH stoichiometrically, thus allowing glycolysis to proceed.

4. The tricarboxylic acid (TCA) cycle

Pyruvate enters the TCA cycle via acetyl CoA the point at which lipids also enter this cycle. The conversion of pyruvic acid to acetyl CoA by an oxidative keto carboxylase requires five cofactors including coenzyme A which is acetylated and NAD^+ which is reduced. Acetyl CoA is then condensed with oxaloacetic acid by a condensing enzyme to form citric acid. The enzyme

aconitase then catalyzes the asymmetric dehydration of citric acid to cis-aconitic acid and subsequent stereospecific hydration to isocitric acid. In the dehydrogenation of isocitric acid to oxalosuccinic acid NAD^+ is reduced. Another closely associated dehydrogenase then decarboxylates the oxalosuccinic acid to alpha-ketoglutaric acid. Following this alpha-ketoglutaric acid is oxidatively decarboxylated to succinic acid in a complex series of rections in which FAD^+ is reduced, CO_2 is released and guanosine triphosphate is produced in a substrate-level phosphorylation. Succinic acid is then oxidized to fumaric acid with the reduction of succinic dehydrogenase. Fumarase catalyzes the addition of a water molecule to fumaric acid to produce malic acid. The final oxidation of the cycle is accomplished by malic dehydrogenase in converting malic acid to oxaloacetic acid and reducing NAD^+. With the acetylation of oxaloacetic acid to citric acid, the cycle is thus completed.

5. Oxidative phosphorylation

It was noted that in various reactions of the Embden-Meyerhof pathway and TCA cycle, proton acceptors notably NAD^+ were reduced. In the respiratory chain electrons are transferred from these substrates through a series of intermediates to reduce oxygen to water. In this series the redox potential of the components also rises from a value of -320 mV for NADH at pH 7 to +820 mV for oxygen. The energy lost from the components may be stored in high energy compounds by, for example, the phosphorylation of ADP and a part of it is lost as heat. Thus the points in this series of coupled reactions where the conservation of energy is theoretically possible are those where the energy difference between

successive intermediates is greater than the energy necessary to phosphorylate ADP (theoretically 150 mV for a phosphate transfer potential of 7 k cal/mole). Although it would be theoretically possible to phosphorylate seven ADP molecules based on observed redox values and the free energy of hydrolysis of ATP, the respiratory chain contains only three points where the redox potential change exceeds 150 mV and, thus, only three molecules of ATP are actually formed as a result of the overall process. The specific points at which the redox potential change ($\Delta E'_o$) enables phosphorylation of ADP are between NAD and flavoprotein ($\Delta E'_o$ = 260 mV), between cytochrome b and c ($\Delta E'_o$ = 260 mV) and between cytochrome c and oxygen ($\Delta E'_o$ = 530 mV).

6. Energy balance-efficiency

An important aspect of energy transformations is the efficiency with which they operate. The complete combustion of glucose to carbon dioxide and water yields about 686,500 cal/mole and this represents the maximum theoretical energy that could be conserved in metabolism. The amount actually conserved is considerably less. In glycolysis two ATP molecules are utilized in phosphorylating glucose and fructose-6-phosphate so that, of the total of 4 ATP formed directly, the net energy conserved in this pathway anaerobically is 2 ATP. Since NADH, when oxidized aerobically yields 3 ATP the reduction of 2 NAD^+ per mole glucose in this pathway makes possible the formation of an additional 6 ATP, bringing the total of ATP formed in glycolysis to eight.

Most of the energy conserved from the catabolism of glucose is made available from the oxidation of coenzymes reduced in connection with the

operation of the TCA cycle. Since four molecules of coenzymes (three NAD^+ and one FP) are reduced and one GTP is formed per turn of the cycle and two cycles are required to oxidize the products of one molecule of glucose, the net yield is 22 ATP and 2 GTP or the equivalent of 24 ATP per mole. In addition, the conversion of pyruvic acid to acetyl CoA reduces NAD^+, which on oxidation yeilds six additional ATP.

Thus in the overall process a net total of 38 ATP are formed in the biological oxidation of glucose to carbon dioxide and water. If the heat of hydrolysis of ATP is taken to be 7 k cal per mole ATP, the total energy conserved is 266,000 calories with an efficiency of about 40%. The above value for the energy conserved in ATP, however, is probably low (Lehninger, 1965) and, if corrections are made for intracellular concentrations of ATP, ADP, inorganic phosphate, Mg^{++} and for the fact that cells are open systems, a more reasonable approximation would be 11.3 k cal/mole ATP (Bartley et. al., 1968) with efficiency of about 63%.

The observations that smooth muscle produces a large amount of lactate aerobically (Kirk, 1963) with a relatively small oxygen uptake suggest that in smooth muscle relatively little of the pyruvate produced in glycolysis is oxidized by the mitochondria. Thus the efficiency of smooth muscle metabolism is substantially less than the maximal value cited above.

7. Role of high energy phosphates

i. Introduction

The preceding section has anticipated the central role of high energy phosphates as the energy storage system of the cell. They have achieved this

position because 1) of the fact that energy per se is a very labile entity and temperature gradients are negligible in biological tissues, so energy must be stored in chemical bonds and 2) in order to achieve an efficient transfer of energy from one chemical reaction to another it is necessary to have a common intermediate (West et. al., 1966). It is thus clear that if this common intermediate contains enough energy to drive the second reaction energy coupling is achieved.

It is important to note that although it is common to speak of high energy bonds, it is not in fact the bonds that contain the energy but rather the energy is derived from the ability of the molecule to derive greater conformational stability with a lower potential energy when certain restraining bonds are broken. Thus, for example, when the terminal phosphate of ATP is transferred to another molecule the transfer will be accompanied by a decrease in the free energy of the donor molecule (Lehninger, 1965).

The phosphate transfer potential or free energy of hydrolysis ($\Delta G''$) of various biologically important phosphates is determined by their relation to the rest of the molecule. The transfer potential is important in that it enables prediction of the phosphorylation reactions which will be energetically favoured.

It is also important that the donor phosphate molecule in the mainstream of biosynthesis have an intermediate phosphate transfer potential since it must be rephosphorylated from higher energy phosphates and yet contain sufficient energy to drive most of the important cellular synthetic reactions. Furthermore it should not itself be an intermediate in metabolism for

it would then be tying up potential energy rather inefficiently. Thus the intermediate phosphate transfer potential of ATP gives it a unique position with reference to energy transfer.

There are other nucleotide-sugar-phosphate combinations that can and do take part in biological phosphorylations. Guanosine, ionosine, and cytosine can be made to form GTP, ITP and CTP and there is no theoretical reason for ATP to be preferred to any of these others, except that many of the enzymes which make reactions possible have a specific ATPase activity.

We note also that ADP contains a considerable phosphate transfer potential the energy of which, however, cannot be directly utilized by the muscle cell. The myokinase-catalyzed reaction is of considerable importance under conditions of acute energy shortage in that this enzyme catalyzes the reaction:

$$2\ ADP \longrightarrow ATP + AMP$$

and whose equilibrium is very AMP-sensitive.

The primary store for the rephosphorylation of ADP, however is creatine phosphate and, indeed, the pool of creatine phosphate acts as a buffer pool of high energy phosphate to maintain ATP stores. The high energy groups of these compounds are readily transferable through the Lohmann reaction with the direction of the phosphorylation reaction being critically pH-sensitive. The phosphorylation of ADP is favoured at a pH slightly more acid than that at which the phosphorylation of creatine is favoured (Bartley et. al., 1968). These relationships are of advantage to the muscle cell since lactic acid produced in intense activity will cause an intracellular acidosis which, in turn, will favour the phosphorylation of ADP. Conversely, at rest, with the return to normal pH the equilibrium will be shifted to favour the resynthesis of creatine phosphate. This is of additional importance in that a

high ATP concentration is a potent inhibitor of energy metabolism.

The most obvious use for this energy in muscle cells, of course, is for contraction. However no less important to the function of the cell are the ion transport mechanisms for Ca^{++} as well as Na^{+} and K^{+} all of which are involved in excitation-contraction coupling, and the various synthetic processes which maintain the patency of the cell.

We shall now consider the role of energized phosphate stores in the normal and abnormal functioning of skeletal, cardiac and smooth muscle.

ii. Skeletal muscle

Investigators had assumed since shortly after the work of Lundsgaard (1934) who demonstrated muscle contraction after the inhibition of glycolysis with iodoacetate, that the energy source for contraction was ATP. Although early reports had implicated CP in this regard it was subsequently shown by Lohmann (1934) that CP was stable unless a cofactor (ADP) was also present. Since it was demonstrated that myosin also has ATP-ase activity and that ATP supplied that energy for a wide variety of endergonic biochemical reactions it was widely assumed that ATP was the energy source for muscle contraction as well, in spite of a lack of direct experimental support. Additional indirect support, however, was accumulated by investigators working with glycerol-extracted muscles and actomyosin solutions (Szent-Gyorgyi, 1951; Weber, 1958) as they found that ATP could cause the extracted muscles to shorten and to cause actomyosin solutions to associate.

The real problem was encountered in the direct demonstration of ATP

splitting in normal contraction. Although Mommaerts and Rupp (1951) reported the predicted change in 1951, their studies could not be repeated by Fleckenstein, Janke, Davies and Krebs (1954) and Lange (1955) in the middle 1950's. It was thought that perhaps the fact that ATP stores could be shown to decline in rigor (Bendall, 1960) was a false lead. Thus began a frustrating four year search for some other energized compound which could be responsible for driving the contractile machinery. Failing in these efforts the experiments demonstrating a breakdown of CP in a large number of contractions were repeated and confirmed by Mommaerts et. al. (1962). Attempts to demonstrate an increased turnover of ATP due to contraction with isotope tracers (Sacks and Clelland, 1960) were inconclusive, however, since multiple reactions proceed simultaneously.

Another complicating factor of course, was the extremely rapid freezing techniques necessary to prevent stimulation of skeletal muscle in the process of freezing. Freezing the muscle in isopentane or Freon pre-cooled with liquid nitrogen however was found to be adequate. But even so, the amount of ATP calculated to be used in a twitch was very small in relation to the total stores present and if the analysis was done after a tetanus, it could not be determined whether the ATP had been used in contraction or recovery.

It thus became clear that the solution to the problem lay in finding a specific inhibitor of the enzyme ATP: creatine phosphotransferase (CPK). The first indication that such a compound might be available came in 1959 when Kuby and Mahowald (1959) reported that 2,4 dinitrofluorobenzene (DNFB) inhibited purified CPK and myokinase. Davies et. al. (1959) after applying

this substance to rectus abdominis muscles found that the muscle could now perform only three loaded contractions in spite of the fact that energy stores in CP and glycogen were sufficient for a greater number. Subsequent analysis showed that while production of inorganic phosphate production was unchanged, the free creatine concentration did not rise significantly but the ATP concentration decreased proportionately to the amount of work done. When the muscle was subsequently extracted it was shown (Infante and Davies, 1965; Infante et. al., 1964) that CPK was completely inhibited, MK was 30% inhibited and calcium activated ATPase activity was somewhat increased. They also observed complete inhibition of oxygen uptake by these muscles, thus excluding regeneration of ATP from this source. The results also suggested that glycolysis was uncoupled, for although lactate production from glycogen was not inhibited the muscle could not replenish its energy stores if given time to do so. Furthermore, they observed that if a muscle stimulated to exhaustion was given a 10 minute rest it could develop one more contraction although the total ATP store was not increased. The muscle eventually developed rigor after a one hour rest.

These results also give indirect evidence of compartmentalization of ATP, for time appeared to be necessary to allow diffusion from a storage site to a site where it is available for contraction. Additional evidence in this regard is the report by Heldt et. al. (1965) that a space in mitochondria comparable to the cristae-space is permeable to exogenous adenine nucleotides and that the exchange of endogenous nucleotides, (probably in the matrix-space) with the cristae-space, through a postulated translocase can be inhibited with atractyloside. The hypothesis could be easily

tested by treating DNFB-poisoned muscles with atractyloside and observing the efficacy of the 10-minute rest in restoring contractile function.

The next question concerns the stoichiometry of the changes found. Infante and Davies (1962) reported a breakdown of 0.22 μmole ATP/gram in the contractile phase of a sartorius twitch or 0.43 μmole in the whole contraction cycle while creatine contents were constant. Mommaerts and Wallner (1967) in extending these studies to include the breakdown products of ATP, although unable to repeat Davies findings with reference to an ATP utilization in relaxation, demonstrated a liberation of 0.36 μmole Pi, 0.14 μmole ADP, 0.07 μmole AMP and a net diminution of 0.27 μmole ATP. They thus achieved a very good stoichiometric balance.

The use of DNFB to elucidate the energy utilization of muscular contraction has, however, been the focus of some criticism. As was mentioned above, DNFB is not absolutely specific for CPK but inhibits myokinase as well, inhibits oxidative phosphorylation and probably uncouples glycolysis. While those actions would certainly place some limits on experimental design they would not necessarily limit its usefulness as a tool in studying energy requirements of contraction. Maréchal and Beckers-Bleukx (1966) and Aubert (1964) have raised the much more serious criticism that DNFB alters in some way the energetics of contraction such that the amount of ATP necessary for contraction and maintenance heat of contraction are decreased with increased poisoning. Thus they conclude that the poisoned muscle is a more efficient mechanochemical transducer - a very unlikely situation. However if one recalculates the ratio of $P_o L_o/M$ that they

give for two levels of poisoning given (fig. 2 in Maréchal and Becker-Bleukx, 1966)and correlates these values with the amount of ATP splitting documented in Table 3 of the same paper, one finds that the ratio $ATP/P_o L_o/M$ is a constant which applies to both muscles.

Their criticism appears to be based on the observation of a slight residual CP-breakdown in lightly poisoned muscles and that this phenomenon contributes to the ATP store available. Dydynska and Wilkie (1966) in commenting on the phenomenon observe that the mechanical activity of muscles which showed some residual PC breakdown was not influenced by this activity. They state (p 766) "... (poisoned muscles with residual CPK activity) showed as great a tendency as any others to have diminished twitch tension". Thus the findings of Maréchal and Beckers-Bleukx (1966) are consistent with the hypothesis that CP breakdown after DNFB does not contribute to the utilizable store of energy for contraction. Their heat records are not available for comment. In support of the idea that the contractile process itself is essentially normal after DNFB treatment is Dydynska and Wilkie's (1966) observation that a normal force-velocity relationship, Fenn effect and shortening heat can be demonstrated. They conclude, however, that with repeated contractions many recovery processes are operating simultaneously as evidenced by an abnormally high resting heat production and deamination of AMP, and thus it is impossible to draw up an energetic balance sheet under these conditions.

iii. Cardiac muscle

The relationship of energized phosphate stores to contraction has been of particular interest as it applies to cardiac muscle. Numerous attempts have been made to link these stores to the action of inotropic drugs and the mechanism of the various manifestations of heart failure.

Since the aim in studying experimental models of heart failure is to discover mechanisms which may be operative in producing the spontaneous lesion, it might be well to consider briefly the applicability of various models which have been studied. Most causes of failure of the heart in vivo can be attributed to either a prolonged increase in work load or a defect in metabolism. Cardiac work, the product of pressure and volume, can be increased by an increase of either of these two factors. Heart muscle, as is true of muscle generally, responds to the increased work load by hypertrophy and this factor may prove detrimental to cardiac performance through distortion and destruction of the sarcomere structure by undue stretching (Sonnenblick et. al., 1964). Studies showing decreased myofibrillar ATPase activity (Chandler et. al., 1967) decreased contractility of isolated myofibrils (Kako and Bing, 1958) and heart failure with normal energy stores (Pool et. al., 1967) suggest that distortion may physically cause suboptimal interaction of the contractile proteins.

Although numerous studies have been undertaken with a view to demonstrating a relationship between energized phosphates and congestive heart failure, the role of energy supplies in the lesion has not been established. Olson and Piatnek (1959) could not demonstrate any decrease in ATP or CP in

congestive heart failure or thyrotoxicosis. Feinstein (1962), with improved techniques was able to show not only that the absolute amounts of ATP and CP in controls were four times the values reported by Olson and Piatnek (1959) but that CP and ATP were reduced by about 50% and 20% respectively in congestive failure. Schwartz and Lee (1962) claimed that mitochondria from hearts in congestive failure were uncoupled and had a depressed respiratory rate. Subsequent studies, however, with oxygen electrodes (Sobel et. al., 1967) have shown that these mitochondria have normal P/O ratios, respiratory control, oxygen consumption and ATPase activity. A number of other studies (Pool et. al., 1967; Furchgott and Lee, 1961; Furchgott and de Gubareff, 1958) have shown that energy supplies under these conditions are essentially normal with, if anything, a slight decrease in CP content. Since papillary muscle from the hearts also shows this slight deficit but can correct it in favourable circumstances in vitro (Pool et. al., 1967) these authors conclude that any depression of high energy phosphate reserves represents an imbalance between energy production and utilization rather than a primary defect in metabolism.

A wide variety of other experimental conditions and drugs which affect the performance of cardiac muscle also act without producing significant changes in cellular energy levels (Furchgott and de Gubareff, 1958). Epinephrine, for example, at low concentration has little effect on ATP and CP, but, at high concentration, decreases ATP and CP while cyclic AMP is transiently increased (Williamson, 1966). Cardiac glycosides, acetylcholine, the Bowditch effect and low calcium have all been shown not to produce any

change in ATP and CP levels (Furchgott and de Gubareff, 1958).

In contrast to the unremarkable effects of the above models of heart failure on energy supplies, hypoxia of cardiac muscle produces an immediate fall in CP followed by a more gradual but progressive decrease in ATP (Furchgott and de Gubareff, 1958). These findings have been established in numerous studies (Feinstein, 1962; Lee et. al., 1961) and are also typical of the effects of metabolic inhibitors. Stoichiometric balance between decreases in ATP and CP and increases in ADP and AMP is not attained, however, since AMP is deaminated with the resultant formation of inosine and hypoxanthine (Imai et. al., 1964). This is of obvious benefit to the cell since the myokinase equilibrium is very sensitive to the AMP concentration.

An interesting observation on the recovery of cardiac muscle from hypoxia is that the return of mechanical tension correlates very well with the recovery of CP stores (Furchgott and de Gubareff, 1958). This has led to speculations (Feinstein, 1962) not consistent with the relationships of ATP and CP to contraction which has been documented in skeletal muscle. The experimental results, both for loss of tension development and its recovery after hypoxia are consistent with the possibility that the ATP available for contraction represents a small compartment characterized by a high turnover rate (Opie,1969a). A similar conclusion has been reached for skeletal muscle by Hohorst et. al. (1962).

iv. Smooth muscle

Since the basic mechanism of contraction of smooth muscle is assumed to be little different from that of cardiac and skeletal muscle it is not surprising

to find that its energy stores are, again ATP and CP and that they are formed largely from anaerobic and aerobic carbohydrate metabolism.

The absolute size of the energy stores, however, while exhibiting considerable variation from muscle to muscle has been shown to be much smaller than in cardiac and skeletal muscle (Furchgott, 1966). Actual values for ATP and CP range from 0.91 and 0.4 - 0.7 μmoles per gram (wet weight) respectively in mesenteric arteries (Beviz et. al., 1965) to 1.8 and 1.94 μmoles per gram respectively in taenia coli (Born, 1956). If one might be permitted a generalization on the few figures reported for energy stores in smooth muscle, it would appear that phasically contracting single unit smooth muscles such as taenia coli and uterus contain greater energy supplies than do tonically contracting multiunit muscles such as arterial and trachealis muscle.

Smooth muscle also behaves predictably in anoxia and metabolic poisoning (Born, 1956; Lundholm and Mohme-Lundholm, 1962) with CP decreasing to a greater extent than does ATP. It appears that the isometric force of contraction of smooth muscle, like cardiac muscle, reflects CP stores rather than those of ATP (Born, 1956; Csapo, 1960).

The effect of drugs on the energy content of smooth muscle has not been thoroughly studied. Adrenaline and acetylcholine have been reported (Lange, 1955) to increase the ATP content while contracting rabbit stomach muscles. A similar observation has been made for the action of adrenaline on mesenteric arteries (Lundholm and Mohme-Lundholm, 1962) but their CP content was stable.

A most interesting relationship between the energy content of mesenteric arteries and the energy cost of contraction has been demonstrated by Lundholm and Mohme-Lundholm (1962 a, b, 1965). Stimulating these muscles anaerobically with adrenaline, they measured both the increase in lactate content (6.8 μEq/g wet weight) and the decrease in ATP and CP content (0.7 μEq/g). On the basis of one mole of ATP synthesized per mole lactate produced it was calculated that 7.5 μEq/g of high energy phosphates had been consumed in the contraction over and above basal metabolism. Since this value is over three times the total preformed store of these compounds, this would imply that smooth muscle is dependent on a continuous energy production for its contractile activity. In this we see a fundamental difference in the relationship of contraction to metabolism between smooth muscle and skeletal muscle, which can be calculated to use only 3% of its preformed energy supplied for a maximal contraction. Insofar as the time course of tension development in the two muscles is quite different the adrenaline contracture requiring 15 minutes and a skeletal muscle tetanus less than 1 second to reach maximum tension, the two are not strictly comparable. The phenomenon would,however, appear to be of sufficient interest to investigate its implications in the physiology of other smooth muscles and with agonists which have no independent metabolism-stimulating properties.

II. Energy metabolism in smooth muscle

a. Introduction

Most of our present knowledge of biochemical pathways in smooth muscle is based on the striated muscle model and on the results of investigations

concerning vascular smooth muscle and the progress of atherosclerosis (Kirk, 1963; Zemplényi, 1962). While the data on the various pathways is still incomplete the key enzymes of most routes of metabolism have been demonstrated in smooth muscle. These include routes for energy liberation from non-carbohydrate substrates such as fatty acids, ketone bodies and amino acids. The glycogenolytic, and glycolytic pathways which produce ATP at the substrate level have also been documented in some detail and these form the basis of our understanding of anaerobic metabolism. Most of the enzymes of the tricarboxylic acid cycle have been demonstrated and, although the quantitative significance of oxidative phosphorylation appears to vary among smooth muscle preparations, satisfactory mitochondrial preparations have been obtained from smooth muscle. Although fewer in number and of smaller activity, they appear to be qualitatively similar to those from skeletal and cardiac muscle. Evidence for a functional HMP shunt and sorbitol pathway has also been obtained in smooth muscle. The evidence for these pathways in smooth muscle is reviewed below.

A possible factor in the energy balance achieved by the muscle under various oxygen concentrations is the ATPase activity of its various components. The total activity could be measured either by measuring the rate of utilization of high energy phosphates in a metabolically poisoned muscle or by measuring the ATPase activities of the various cell fractions. While these activities have been measured for various cell fractions (Needham and Schoenberg, 1967) no data exist on whether these are influenced by hypoxia.

The energy utilization by resting and stimulated smooth muscle has also not been determined, but it is certainly greater than that produced solely by

glycolysis. Thus in hypoxia, with the mitochondria and, thus, energy production from non-carbohydrate sources inhibited, glycolytic flux is increased through the stimulation of phosphorylase b activity by rising AMP levels. In this way a partial compensation for inhibited oxidative pathways may be effected.

b. Glycogen metabolism

The pathway by which glycogen is synthesized in smooth muscle is controversial. Bo and Smith (1965) demonstrated the presence of UDPG-glycogen synthetase in intestinal smooth muscle histochemically. Takeuchi and Glenner (1961), however showed that its activity is small in comparison with that of phosphorylase and these findings led Adams (1967) to conclude that in smooth muscle only the phosphorylase route is utilized in glycogen synthesis and degradation. It is probable, however, that artifacts of the fixation technique (see comments below) activated phosphorylase and, thus, overestimated the normal phosphorylase activity in living tissue. Other authors (Kirk, 1963) assume the UDPG-glycogen synthetase pathway is operative in glycogen synthesis. In view of the objection raised with reference to the synthesis of glycogen by phosphorylase in skeletal muscle (reviewed above) namely that the reactants, glucose phosphates never occur in tissues in concentrations high enough to favour glycogen synthesis, it would seem unlikely that this condition could be met in smooth muscle either.

The pathway for glycogenolysis in smooth muscle, that catalyzed by phosphorylase is well established, with the presence of this enzyme being demonstrated both histochemically (Takeuchi and Glenner, 1961) and

enzymatically (Kirk, 1962; Diamond and Brody, 1966; Bueding et. al., 1962). The associated enzyme necessary to both routes of glycogen metabolism, phosphoglucomutase, has also been demonstrated in human arterial muscle by Kirk (1963).

The demonstration of the activation of phosphorylase in conditions known to produce increased glycogenolysis has been difficult, with changes smaller than expected owing in large part to the high resting phosphorylase a activities measured (Mohme-Lundholm, 1962). Studies (Namm, 1967; Bueding et. al., 1962; Diamond and Brody, 1966) using techniques designed to stop cellular metabolism more rapidly (Hardman et. al., 1965) have, however, demonstrated a phosphorylase a activity as a fraction of the total phosphorylase activity comparable ($< 10\%$) to that in skeletal muscle with a notable activation in response to various stimuli including phenylephrine and K^+-depolarization. It is important to note, however, that the total phosphorylase activity of smooth muscle (4.6 μmole Pi/min; Diamond and Brody, 1966) is about 6% of that of skeletal muscle (72 μmole Pi/min; Danforth et. al., 1962). This relatively small activity in smooth muscle is consistent with the low rate of contraction and consequently smaller energy utilization rate.

c. Glycolysis

The enzymes of glycolysis have been investigated systematically by Kirk and co-workers and have been found, generally to be present in a small fraction (3-10%) of the concentrations found in skeletal muscle (Sanwald and Kirk, 1966 a, b). Nevertheless differences in activity (expressed as

m mole substrate/g wet tissue/hr in this discussion) of individual enzymes may reveal the rate-limiting steps in the pathway. Hexokinase, for example, has an activity (0.013) of only about 1% of that of phosphoglucoisomerase (1.85) in arterial muscle (Brandstrup et. al., 1957), the latter enzyme being the most active of any investigated in smooth muscle. The activity of phosphofructokinase (0.00408),on the other hand, is about 0.3% of that of phosphoglucoisomerase (Ritz and Kirk, 1967) and it is probably a central rate-limiting step in glycolysis in smooth muscle as it is in skeletal muscle. The activity of aldolase (0.056 m mole substrate/g wet tissue/hr) is higher (by a factor of 14) (Kirk and Sørensen, 1956) and that of glyceraldehyde phosphate dehydrogenase (0.167) is higher still (Kirk and Ritz, 1967). Kirk and Ritz (1966) also demonstrated the presence of phosphoglyceric acid kinase. The relatively high activities of enolase (0. 24; Wang and Kirk, 1959) and lactic dehydrogenase (1.06; Kirk et. al., 1958) may be of importance in view of the predominance of glycolysis, the former because of the phosphorylation of ADP and the latter because of the low fraction of glycolytic end products that are further oxidized in the mitochondria of smooth muscle.

Some associated carbohydrate pathways have also been found to be operative in smooth muscle. The finding of a fairly high phosphomannose-isomerase activity in arterial tissue (Kirk, 1963) is interesting since this allows the metabolism of mannose-6-phosphate in glycolysis. Evidence for an active sorbitol pathway which converts sorbitol to fructose has also been

reported by Ritz and Kirk (1967), though its quantitative physiological significance has not been established.

d. Tricarboxylic acid cycle

The enzymes aconitase (0.021; Kirk, 1961) isocitric dehydrogenase (0.063; Kirk, 1960) succinic dehydrogenase (0.035; Kirk et. al., 1955) fumarase (0.178; Sørensen and Kirk, 1956) and malic dehydrogenase (0.57; Matzke et. al., 1957) of the tricarboxylic acid cycle have been demonstrated and measured in arterial muscle. Of these, the activity of aconitase is comparatively low, though the metabolic significance of this observation has not been established.

e. Hexose monophosphate shunt

The importance of this pathway lies in its production of 1) TPNH which is necessary for the synthesis of fatty acids and steroids (e.g. cholesterol) and 2) pentose phosphate compounds for the synthesis of nucleic acids and nucleotides.

The enzymes glucose-6-phosphate dehydrogenase (0.115), 6-phosphogluconate dehydrogenase (0.010) and ribose-5-phosphate isomerase (0.0034) have been demonstrated to be present in arterial tissue in progressively diminishing amounts (Kirk, 1963) though transketolase is more active. Nevertheless, Sbarra et. al., (1960) in measuring metabolic flux with radioactive tracer techniques have demonstrated an important function of this pathway in guinea pig aorta. Since this pathway relies on oxygen as the final acceptor of electrons from the oxidation of TPNH, it cannot function in hypoxia.

f. Lipids and amino acids in energy metabolism

It is well known that resting striated muscle relies largely on oxidative lipid energy metabolism and it is interesting , in view of the importance conventionally

ascribed to carbohydrate metabolism to note that amino acids and lipids as fatty acids may be utilized to a considerable extent in the oxidative metabolism of smooth muscle as well. Measurement of glutamic dehydrogenase (0.012; Kirk, 1965) and the enzymes alpha-hydroxybutyric dehydrogenase (0.329; Sanwald and Kirk, 1966 b) and beta-hydroxyacyl-CoA dehydrogenase (0.013; Sanwald and Kirk, 1965) of lipid metabolism as well as the demonstration by Furchgott (1966) that smooth muscle depleted of endogenous substrate will recover when given butyrate or acetate have established that these pathways can be of quantitative importance to smooth muscle function. Since these substrates can be utilized only in oxidative metabolism, however, their contribution in hypoxia is not important. The question of which substrates are utilized by smooth muscle in vivo is not settled yet but Furchgott's (1966) work and the observation (Stephens and Wrogemann, unpublished observation) that smooth muscle mitochondria can utilize palmitate (with carnitine) suggests that these substrates are of greater importance than has been generally recognized.

g. Oxidative phosphorylation

Until recently studies of smooth muscle mitochondria were hampered by the fact that good mitochondrial preparations could not be obtained. Not only were mitochondrial yields from pig uterus low, but P/O ratios of less than 1 and low phosphorylating capacities were reported by Gautheron et. al. (1961). Wakid (1960) was also not able to obtain a good mitochondrial preparation from rat uterus and, in fact, no oxidative phosphorylation could be detected though an ATPase activity was demonstrated. Clearly, no conclusions on mitochondrial function could be drawn from these studies. Recently Stephens and Wrogemann

(1970) have isolated mitochondria from tracheal smooth muscle which had respiratory control ratios of 4.5 and ADP/O ratios of 2.3 with pyruvate-malate as substrate. Thus these mitochondria are similar to those from skeletal and cardiac muscle. The quantity of mitochondrial protein and oxygen uptake rates, however, were smaller, resulting in a phosphorylation capacity of about 10% of that in skeletal muscle. These results suggest that oxidative phosphorylation produces a smaller proportion of energy for contraction in smooth muscle than in skeletal muscle.

h. Conclusions

Although it is clear that many of the observations on smooth muscle biochemistry have particular pertinence for vascular smooth muscle, it seems that normal oxidative metabolism in smooth muscle is no less complex in smooth muscle than in striated and cardiac muscles. While carbohydrate metabolism may well be the major source of energy in normal circumstances, evidence justifying this widely held assumption is not conclusive. With the imposition of hypoxia ($P_{O_2} < 10$ mm Hg) as an experimental condition, however, all non-glycolytic metabolic pathways are inhibited.

Since 1) the rate of active processes is affected by changes in high energy phosphate concentrations and 2) the regulation of the activation of muscle depends on actively maintained ion gradients, most notably for Ca^{++}, the role of Ca^{++} in muscle contraction is reviewed below.

C. ROLE OF CALCIUM IN MUSCLE CONTRACTION

I. Striated muscle

a. Introduction

The central role of calcium ions (Ca^{++}) in the contractile process of muscle has been appreciated since the time of Ringer's (1883) classical experiments demonstrating that the presence of this ion was crucial to the continued beating of isolated frog hearts. Numerous subsequent studies on various muscle extracts, model systems and living muscle fibres indicated the possibility that ionic calcium could activate processes related to contraction. On the basis of those observations Sandow (1952) proposed that "in living muscle, activation of the contractile material...may be attributed to the enzymatic activation of the myosin-ATPase system by Ca^{++}" and suggested a model in which the action potential of muscle caused a calcium influx which in some way activated the contractile process. A rigorous proof of this hypothesis, however, required the demonstration that a) calcium ions injected into a muscle cell cause a local contraction, b) sufficient calcium enters the fibre in a single action potential to maximally activate the contractile proteins, c) the diffusion time for Ca^{++} from its site of entry to the contractile elements is sufficient to account for the time of onset of contraction, d) removal of Ca^{++} from the surface of muscle fibres abolishes the contractile response to stimulation (Frank, 1965), e) removal from the sarcoplasm precedes relaxation and, f) specific proteins of the contractile apparatus extracted from muscle

have Ca^{++} binding properties in the physiological range of Ca^{++} concentrations and that, associated with Ca^{++}-binding enzymatic (ATPase) and physico-chemical changes take place which are consistent with the idea of contraction. We shall now review the evidence bearing on the various aspects and predictions of this hypothesis in striated muscle as it forms a basis for our understanding of phenomena in smooth muscle.

b. Evidence for role of Ca^{++} from studies on:

1. Intracellularly-injected Ca^{++}

Heilbrunn and Wiercinsky (1947) were the first to demonstrate the contractile response of skeletal muscle fibres on the microinjection of Ca^{++} at low concentration. This was the only cation species of the several occurring physiologically which would cause this effect. These findings were confirmed by Niedergerke (1955) and extended to show a similar response of skinned muscle fibres to applied Ca^{++} (Podolsky and Costantin, 1964). Furthermore, by using this technique contractions of half as well as whole I-bands could be observed and it was clear that in these experiments finer gradations of contraction could be elicited than with an action potential at the membrane. This evidence supported the notion that Ca^{++} release follows membrane excitation in the sequence of its coupling to contraction. It was also observed that the contraction of both halves of the I-band was symmetrical when Ca^{++} was injected at the Z-line but that only the half I-band adjacent to the site of injection in the A-band contracted. Thus Huxley and Taylor's (1958) observation of symmetrical contraction of I-bands

with local depolarization at the Z-line could be interpreted in terms of symmetrical Ca^{++} release from the region of the Z-line.

Work on models of muscle contraction have supported the evidence cited above. Studies on glycerinated muscle preparations whose contractile proteins are operational but whose membranes and biochemical pathways are non-functional have shown that, in the presence of Mg-ATP, contraction is initiated by the introduction of critical amounts of Ca^{++} to the suspension medium. Gillis (1969) has, moreover, demonstrated that in these preparations diffusion of calcium to the actin-myosin overlap region of the A-band was crucial to force generation regardless of the length to which they were stretched.

2. Calcium influx during depolarization

The net influx of Ca^{++} during action potentials and depolarization was demonstrated in frog sartorius muscle by Bianchi and Shanes (1959) in cardiac muscle by Niedergerke (1959) and in squid giant axon by Hodgkin and Keynes (1957). The correlation of the duration of increased Ca^{++} influx with the duration of high-potassium contractures (Shanes, 1961) as well as the inotropic actions of nitrate ions with increased calcium influx are additional circumstantial evidence of a possible causal relationship between calcium influx and contraction. Although the increased Ca^{++} influx could be readily demonstrated during the action potential, the critical factor with reference to Sandow's model was the amount of Ca^{++} entering. Bianchi and Shanes (1959), in measuring these

fluxes showed, however, that the amount of Ca^{++} entering fibres was much less than one Ca^{++} per myosin and, thus, could not be the main activator of the contractile process. These studies, however, did not measure the intracellular $[Ca^{++}]$ and, thus, did not rule out the possibility that intracellular calcium was being mobilized by the depolarization to initiate contraction.

3. Latent period and the diffusion time of Ca^{++}

Another serious objection to the hypothesis on Ca^{++} influx proposed by Sandow was the observation by Hill (1949) that muscle becomes fully activated throughout the cross-section of the fibre in its latent period. When the diffusion time for the Ca^{++} ion to reach the center of the fibre from the sarcolemma was calculated, the value obtained was too long to account for the rapid activation of muscle. Sandow, of course, recognized this objection and postulated an exchange diffusion which might be operative to accelerate the process. Numerous subsequent studies, however, have demonstrated the presence of a transverse tubular system continuous with the sarcolemma (Porter and Palade, 1957) and open to the extracellular fluid (Huxley, 1964; Peachey and Schild, 1968) which is capable of conducting electrical impulses to the interior (Huxley and Taylor, 1955, 1958) where associated reticular structures are capable of releasing (Weber and Herz, 1968; Lee et. al., 1966) and binding (Hasselbach, 1964; Weber, 1966; Ohnishi and Ebashi, 1964) calcium. Recent studies on the nature of Ca^{++}-release by these structures (Ford and Podolsky, 1970) indicate

that it may be a regenerative phenomenon triggered by Ca^{++} released in the current of depolarization. The diffusion distance for Ca^{++} is thus reduced sufficiently to permit the rapid activation of muscle in accordance with this hypothesis. Additional indirect support in this regard is evidence that the intracellular calcium concentration does reflect the active state. This has recently been demonstrated with the use of intracellular calcium-specific indicators such as murexide (Jöbsis and O'Connor, 1966) and aequorin (Ridgeway and Ashley, 1967; Hoyle, 1970). Thus it appears the diffusion distances are small enough so that Ca^{++} transients to and from the sarcoplasmic reticular structures could activate contraction with the observed time course and that, in fact, observed calcium transients match those expected for an activator substance.

4. Contractile responses in calcium-free media

If the calcium ion is the excitation-contraction coupling agent it should reasonably follow that depletion of muscle calcium by equilibration in Ca^{++}-free media should abolish contractile responses to stimulation. The fact that this prediction could be experimentally verified in early experiments (Ringer, 1883), however, established only that this ion was essential to the coupling process.

A complicating factor in experiments of this kind was the observation that the effect of calcium deficiency was not solely on activator calcium but that membrane permeability was increased with attendant depolarization. Since the time courses of both phenomena were similar (Frank, 1960) the independent effect of Ca^{++} removal on the coupling process could not be assessed.

In order to obtain a more direct answer to this problem, Frank (1965) placed the muscle in a sodium-free choline-substituted Ringer solution to render the muscle inexcitable and used potassium induced contractures as a model. The observation that in these conditions the potassium response was rapidly eliminated in a calcium-free medium showed that the link in electromechanical coupling could be removed by calcium removal.

5. Velocity of calcium accumulation

Measurements of calcium transients (Jöbsis and O'Connor, 1966; Ashley and Ridgeway, 1968) have shown that the decrease in free intracellular calcium concentration precedes relaxation. The general correlation between the amount of sarcoplasmic reticulum (SR) and the rapidity of muscle contraction (Weber, 1966) when actomyosin ATPase is not rate-limiting (Bárány, 1967) would suggest that this structure might be primarily involved in the regulation of intracellular Ca^{++} concentration through uptake and release mechanisms. Numerous studies have thus been directed to the measurement of the maximal rate of Ca^{++} accumulation by this system to determine whether its activity can account for the rapid disappearance of Ca^{++} and relaxation. Values for Ca^{++} uptake by fractionated reticulum on conventional apparatus (Weber et. al., 1966) were slightly less than half the values required to account for relaxation. Although these values could be reconciled with the observed speed of relaxation by applying suitable correction factors for isolation procedures, it had been observed (Weber et. al., 1963) that suspensions of reticulum formed aggregates continuously and that the stirring rate affects uptake rate measurements and,

thus, the values reported could conceivably be affected by experimental artefact. The measurement of Ca^{++} uptake rates well in excess of the required value by Ohnishi and Ebashi (1964) by implementing a rapid stirring procedure confirms that the Ca^{++} accumulation by this structure can account for the rapidity of relaxation. It is, however, important to note at this point that although it is necessary for the rate of Ca^{++} accumulation by this structure to account for the rapidity of onset of contraction and the time course of relaxation, there is no correlation theoretically necessary between the amount of SR and the rate of contraction, though it usually does exist. A notable exception is the tortoise heart where myosin ATPase activity is rate-limiting in the presence of a well developed SR (Bárány, 1967).

6. Regulation of the interaction of contractile proteins by Ca^{++}

Although there are several conditions in which physicochemical analogs of contraction (syneresis, increased ATPase activity, superprecipitation) may proceed in the absence of Ca^{++} (Weber, 1966), the most important for the study of regulatory mechanisms is that which proceeds when actomyosin is reconstituted from highly purified actin and myosin. The ATPase activity of this preparation is maximal and not Ca^{++}-sensitive in contrast to that of "natural" actomyosin systems. Weber and Winnicur (1961) showed that the crucial difference between these two systems was in the purity of the actin preparation. In further investigating the phenomenon, Ebashi and Ebashi (1964) found that, if a protein fraction closely resembling tropomyosin was added to the synthetic

actomyosin system or to a Ca^{++}-desensitized trypsin-treated natural actomyosin system, full Ca^{++}-sensitivity was returned to the system, with ATPase activity reduced by 85% in the absence of Ca^{++} (Maruyama et. al., 1964). Ebashi's protein, troponin, was found to be bound in a complex with tropomyosin along the whole length of the actin filaments (Ebashi and Endo, 1968; Nonomura, et. al., 1968) and it is this complex which in some way prevents the interaction of actin and myosin (Ebashi et. al., 1968). On the binding of Ca^{++} by troponin, however, a reaction (possibly a conformational change) mediated by tropomyosin releases the inhibition previously effected by the complex and actomyosin interaction takes place (Wakabayashi and Ebashi, 1968). Thus the effect of Ca^{++} appears to be as a releaser of inhibition rather than as an essential link in cross bridge formation as previously proposed by Davies (1963).

Since the amount of Ca^{++} needed for binding to contractile proteins in the activation process was known, it was crucial to the above scheme to determine whether troponin could bind sufficient Ca^{++} to fully activate the contractile system. The relevant parameters were measured by Fuchs and Briggs (1968) and the value for Ca^{++} binding obtained by them was actually somewhat greater than that observed in maximal activation.

Thus the available data support the hypothesis that the intracellular free Ca^{++} concentration regulates the activation of muscle and that structures available for the uptake and release of this ion possess the required apparatus to perform these functions with a rapidity equal to the speed of activation as well as relaxation, though Sandow's (1952) model for Ca^{++} entry was inadequate.

II. Smooth muscle

a. Evidence for Ca^{++} as the activator of contraction

Although the absence of sarcomere units in smooth muscle implies some difference between the nature of contraction in this muscle and that in striated muscle, the demonstration of Ca^{++}-sensitive actomyosin extracted from various smooth muscle (Filo et. al., 1963; 1965; Needham and Williams, 1963) and the presence of a sarcoplasmic reticulum (Schoenberg, 1958; Carsten, 1969) with the ability to accumulate Ca^{++} suggest that the basic mechanism of contraction and the role of Ca^{++} in its activation are similar in the two types of muscle. Additional evidence analogous to that reported for skeletal muscle in this regard is the observation that 1) net Ca^{++} influx has been demonstrated in the electrical stimulation of the taenia coli (Sunnano and Miyazaki, 1968; Lüllmann and Mohns, 1969) and, indeed, recent evidence indicates that in this muscle the current of the action potential is carried by Ca^{++} (Brading et. al., 1969), 2) a relatively large amount of Ca^{++} enters the fibres in forced electrical stimulation, and because of the small size of smooth muscle cells (Lüllmann and Mohns, 1969) and slow time course of contraction (Stephens et. al., 1969) diffusion time for Ca^{++} from the cell surface does not pose the problem in smooth muscle that it does for skeletal muscle and, 3) the link in excitation-contraction coupling can be broken by the removal of calcium from the surface of smooth muscles (Bozler, 1960; Durbin and Jenkinson, 1961). The latter point, however, is more difficult to demonstrate as being unaffected by the concomitant change in excitability

(Marshall, 1965; Bülbring and Kuriyama, 1963 a) in smooth muscle than in skeletal muscle (Frank, 1965) because of the various ways in which smooth muscle may be stimulated (Somlyo and Somlyo, 1968) and different calcium fractions involved (Daniel, 1965). Since the interpretation of many of the studies on the mobilization of smooth muscle calcium stores in different experimental conditions is controversial, with various models of the inter-relationship of these stores being assumed in working hypotheses (Daniel, 1965; Hurwitz, et. al., 1967; van Breemen and Daniel, 1966; Goodford, 1965 b) a separate section is devoted to their discussion. Not all studies on the role of Ca^{++} have, however, been consistent with the conventional view presented above. Isojima and Bozler (1963), for example, observed that the responses of frog stomach muscle to acetylcholine disappeared in a Ca^{++}-free solution but returned in a Ca^{++}-free K^{+}-depolarizing solution and that a net uptake of Ca^{++} was seen in the presence of Ca^{++} in both Na^{+} and K^{+}-containing solutions but that contraction accured only in the K^{+}-solution. Thus they concluded that the response to ACh depends on sensitization of the myoplasm by K^{+} and that Ca^{++} does not directly participate in excitation-contraction coupling in smooth muscle. However, it is well known (Lüllmann and Mohns, 1969; Hurwitz, Joiner and von Hagen, 1967) that net Ca^{++} uptake does not necessarily imply an increase in free intracellular Ca^{++} concentration as Isojima and Bozler's (1963) studies would

suggest. Another study (Sperelakis, 1962) in which strong field stimulation of depolarized cat intestine was observed to increase Ca^{45} and Sr^{89} influx was interpreted to mean that Ca^{++} influx was contraction-dependent rather than polarization-dependent.

Evidence of other fundamental differences between smooth and skeletal muscle has been obtained by Hurwitz and Joiner (1969). The observations that superficial membrane loci could not be saturated (i.e. the fast Ca^{45} compartment was dependent on external Ca^{++} at all concentrations) and that under certain conditions Ca^{++} availability was not limiting to contraction suggested to them that Ca^{++} of the fast compartment was actually in a non-saturable biophase and that a superficial Ca^{++} carrier system which could be saturated was limiting. Stimuli such as depolarization and drug agonists were presumed to act by influencing this carrier system. Though their conclusions are very interesting, their methods are open to some criticism (detailed below - section b-2).

Although the details of the release of activator calcium are not clear, most of the presently available evidence is consistent with the notion that the role of Ca^{++} in initiating contraction in smooth muscle is similar to that in skeletal muscle.

b. Calcium stores in smooth muscle

1. Evidence for multiple calcium stores

A number of physiological observations on the responses of smooth muscle to different stimuli in solutions containing various Ca^{++} concentrations have

suggested the existence of at least two stores of Ca^{++} which can be mobilized to activate smooth muscle. The absence of histological evidence as to the physical inter-relationships of these stores has led to many hypotheses attempting to explain the diverse observations on muscle function in various experimental conditions.

The physiological evidence for the existence of these separate calcium sites, briefly, are the observations that 1) potassium contractures are rapidly abolished in Ca^{++}-free solutions while acetylcholine (or adrenaline) contractures are more resistant to this treatment and these drugs can stimulate K^{+}-depolarized smooth muscle (Hinke, 1965; Edman and Schild, 1962; cf. Waugh, 1962; Robertson, 1960; Hinke, 1964) 2) calcium chelating agents such as EDTA stimulate a contraction in smooth muscle even when applied in high concentrations (Daniel, 1965; Hurwitz, Joiner and von Hagen, 1967 a, b) 3) in aortic muscle the response to adrenaline consists of two phases, one of which (the second or slow phase) is directly dependent on the concentration of extracellular calcium (Bohr, 1963, 1964 a, b) and the fast phase is largely independent of external calcium, 4) the responses of toad stomach muscle to acetylcholine in K^{+}-Ringer are not abolished until tissue calcium is 85% depleted and responses are fully restored by the replacement of only 9% of tissue calcium while the responses to alternating current stimulation are much more Ca^{++}-sensitive (Sparrow and Simmonds, 1965),

and 5) the increased K^+ efflux in guinea pig ileum stimulated by external K^+ and acetylcholine are inhibited by low external $[Ca^{++}]$ and ethanol, respectively but, for example, ethanol has no effect on the K^+-stimulated efflux (Weiss and Hurwitz, 1963). Findings such as these suggest that potassium and electrically stimulated contractions utilize primarily a loosely bound Ca^{++} store, though the observation (Imai and Takeda, 1967) that in Ca^{++}-deficient solutions taenia coli exhibit a tonic and a phasic response to K^+, the tonic response being relatively insensitive to Ca^{++} depletion (10^{-8} M) suggested that K^+ can mobilize some bound Ca^{++} as well. The loosely bound Ca^{++} is probably the membrane store which is dependent on external calcium and membrane polarization. A part of this store may also be involved in the stabilization of membrane permeability since 1) in Ca^{++}-free physiological salt solutions in the absence of Ca^{++} uterine smooth muscle is depolarized (Daniel and Irwin, 1965; Marshall, 1965) though the effect is not noted in non-ionic sucrose solutions (Bozler, 1962) and 2) the rate constant for K^+ efflux is increased in Ca^{++}-deficient solutions (von Hagen and Hurwitz, 1967).

The relative resistance of the effects of agonists such as acetylcholine(ACh) and adrenaline, to external calcium depletion has been interpreted as being a result of the mobilization of tightly bound or sequestered calcium, though in taenia coli ACh may merely increase membrane permeability (Bülbring and Kuriyama, 1963 b). Daniel (1965) has ascribed the

effect of EDTA to the liberation of sequestered calcium as superficial calcium was removed. More direct evidence for the independence of these sites has been obtained by van Breeman and Daniel (1966) in the demonstration that Ca^{45} was released on potassium depolarization whereas they could not observe this effect on treatment with acetylcholine.

2. Models of the inter-relationship of calcium stores

The inter-relationship of these stores has been the subject of considerable speculation (Daniel, 1965; van Breeman et. al., 1966; Hurwitz, von Hagen and Joiner, 1967 a; Goodford, 1965 b). The most esoteric of these was proposed by Goodford (1965 b) to explain calcium fluxes in terms of energy barriers to free diffusion. Although, with four independent and variable barriers, it should be possible to explain almost any phenomenon observed in flux data, the structural and physiological counterparts to some of these barriers are difficult to imagine. He also finds it necessary to postulate active exclusion as well as the active extrusion of Ca^{++} to maintain the high pCa observed intracellularly. However, his emphasis on the physical chemistry of calcium phosphate complexes in what might otherwise be construed as physiological effects is an important point which has been largely neglected in subsequent investigations.

The three simplest conventional arrangements of two calcium stores are 1) series 2) parallel and 3) series/parallel arrangements (Daniel, 1965). The observations that K^+-stimulated contractures are more sensitive to external Ca^{++} depletion than are drug stimulated contractures (Hinke, 1965) and that,

after severe tissue calcium depletion K^+-stimulated contractures are more rapidly restored (Daniel, 1963) would suggest the possibility of a simple series relationship with the loosely bound stores exterior to or more superficial than the sequestered "bound" stores. Goodford's (1965 b) model, however, would imply that the two are in parallel with the above physiological observations accounted for by assuming different rate constants for their calcium loading. Hinke's (1965) observations on the difference in the ability of Ba^{++} to substitute for Ca^{++} in K^+ and adrenaline stimulated contractions, however, suggested that the Ca^{++} mobilized in a K^+ contracture was independent of sequestered calcium. Thus, a series-parallel arrangement was suggested as the one most consistent with observations on the calcium dependence of contraction. The findings of Hiraoka et. al. (1968) are also consistent with this model in that the isolated , perfused vascular system of the rabbit ear responded to norepinephrine in a Ca^{++}-free environment mobilizing presumably bound Ca^{++} while in the presence of external Ca^{++} both Ca^{++} stores appeared to be utilized. The recent demonstration of a functional sarcoplasmic reticulum in uterine smooth muscle (Carsten, 1970) would also be difficult to reconcile with either of the other two proposed models.

The above observations also suggest that, regardless of the particular arrangements of the two sites, the superficial, membrane-stabilizing calcium is relatively loosely bound. Kinetic data, however, are controversial on this point. Van Breemen and Daniel, (1966) found that a

significant Ca^{45} store was still available to be mobilized by potassium up to 120 minutes after removal from the Ca^{45} loading solution. Thus they concluded that its Ca^{++} exchange must be quite slow. In testing this hypothesis, however, they found that it could be labelled by a short (10 min) exposure to Ca^{45}. Since they had no way of distinguishing the fraction of the K^{+} mobilized compartment which was rapidly labelled from that which presumably was not, and since the compartment is in other respects treated as a unit, the experimental basis for their statement that "high K depolarization liberates some superficial Ca, most of which exchanges very slowly and which is in parallel with the remainder of the cellular exchangeable Ca" (p 1303) is not clear. A possible explanation is that in the process of exposing the muscle to Ca^{45} a transient change of temperature or tension on the muscle occured which caused a brief depolarization and allowed the muscle to bind more Ca^{45} in the subsequent repolarization than had been expected from steady state exchange kinetics.

Hurwitz and co-workers have studied the responses principally of guinea pig ileum to acetylcholine and Ca^{++}-free solutions to gather evidence on the nature and inter-relationship of calcium stores. The phenomenon utilized to assess intracellular "bound" calcium stores is the contracture resulting when calcium-loaded (with 36 mM $CaCl_2$) muscles are placed in a Ca^{++}-free solution (Hurwitz, Joiner and von Hagen, 1967 a; Hurwitz et. al.,1969). However the nature of this Ca^{++}-free stimulus is not clear. Daniel (1965)

has suggested that both superficial and bound stores are mobilized to initiate contraction except when very high EDTA concentrations are employed. Hurwitz's argument that since the response to the Ca^{++}-free stimulus increases with tissue calcium content, it is probably cellular calcium that is mobilized does not appear to be cogent as the means used to increase tissue calcium content (high $[Ca^{++}]_o$) probably increases the content of both compartments. The use of isotonic shortening as a measure of activator Ca^{++} also appears to be uniquely unsuitable in this capacity since the degree of shortening in response to a submaximal stimulus is critically load-dependent (e.g. if the load is very small a submaximal stimulus may induce near maximal shortening). Isometric tension development or maximal velocity of contraction would have been better indices of this parameter (Csapo, 1954). Apart from the osmotic shock of adding and withdrawing 36 mM $CaCl_2$, the usefulness of this particular stimulus in mobilizing one calcium compartment selectively and the probability that the mechanical measurements indicate activator Ca^{++} are thus not free of doubt.

The observation that the response to a high dose of acetylcholine was potentiated by a high external Ca^{++} concentration while that of a low dose of this agent was inhibited under these conditions led Hurwitz, von Hagen and Joiner (1967) to propose a model in which calcium in membrane stabilizing sites regulated the release of this ion from a similarly superficial bound store to the cytoplasm. Thus in this model all activating Ca^{++} must pass through the storage site and Ca^{++} from membrane stabilizing sites would have no

independent access to the cytoplasm. Parallel observations by the same authors (Hurwitz, Joiner and von Hagen, 1967 a) however, cannot be explained on the basis of this model. In this paper they demonstrate that the calcium content of muscles contracting equally in response to Ca^{++}-free solution and the combination of acetylcholine plus Ca^{++} (after a prolonged calcium depletion) are different, being less in the latter. If, as is assumed, the exchangeable Ca^{++} is contained in these two stores and, according to the model, activator Ca^{++} loads the storage site en route to the cytoplasm, it is difficult to envision a mechanism whereby "a given increase in tone requires less intracellular accumulation of calcium if an extracellular pool rather than an intracellular pool of calcium supplies the activating ions " as they conclude (Hurwitz, Joiner and von Hagen,1967, p 1302).

III. Conclusions

It has been shown that 1) Ca^{++} is the only physiological cation which, injected intracellularly, initiates contraction 2) sufficient calcium can enter the myoplasm with a time course rapid enough to account for the contractile response and that its removal precedes relaxation 3) the electromechanical coupling can be broken by calcium depletion and 4) the interaction of the contractile proteins is regulated specifically by the Ca^{++} ion in the physiological range of Ca^{++} concentration. Thus the role of the Ca^{++} ion as the

intracellular link in excitation-contraction coupling in skeletal muscle is now well established. Although comparable evidence is not available for smooth muscle, that which is available is strongly suggestive of a basically similar role for this ion in the activation of this muscle as well.

METHODS

A. DISSECTION

The posterior ends of incomplete canine tracheal rings are closed by the paries membranaceus which contains in its most posterior layer the musculus transversus tracheae. This is also called the trachealis and will be so referred to in this communication.

Trachealis muscle was obtained from the cervical tracheae of mongrel dogs anaesthetized with 30 mg/kg pentobarbital (Nembutal, Abbott) iv. The dogs were then killed by injection of saturated potassium chloride solution iv.

Dissection was carried out in the plane of separation between the trachealis dorsally and the tunica fibrosa ventrally. Separation was easy and the muscle layer could be dissected out with minimal handling and trauma. Rectangular strips which were about 1 cm long in the lateral transverse plane of the trachea and about 0.075 cm thick in the anteroposterior plane and 0.1 to 0.2 cm wide in the craniocaudal plane were cut out and placed in a bath containing Krebs-Ringer solution. The composition of this solution is given in Table I. It was equilibrated with an O_2-CO_2 mixture so as to yield a P_{O_2} of 600 mmHg, P_{CO_2} of 45 mmHg and a pH of 7.40, at a temperature of 37° $\pm$ 1° C. Dissection time averaged 3 to 5 minutes and the muscle was kept wet during this time.

B. MECHANICAL STUDIES

The apparatus for mechanical studies was arranged as shown schematically in figure 1 for a single muscle bath. The apparatus used permitted the simultaneous and independent equilibration of six muscle strips. The lower end of

TABLE 1

Composition of Krebs-Ringer

	g./l.	mM
NaCl	6.72	115
$NaHCO_3$	2.10	25
NaH_2PO_4	0.167	1.38
KCl	0.187	2.51
$MgSO_4$	0.296	2.46
$CaCl_2$	0.145	1.91
Dextrose	1.00	5.56

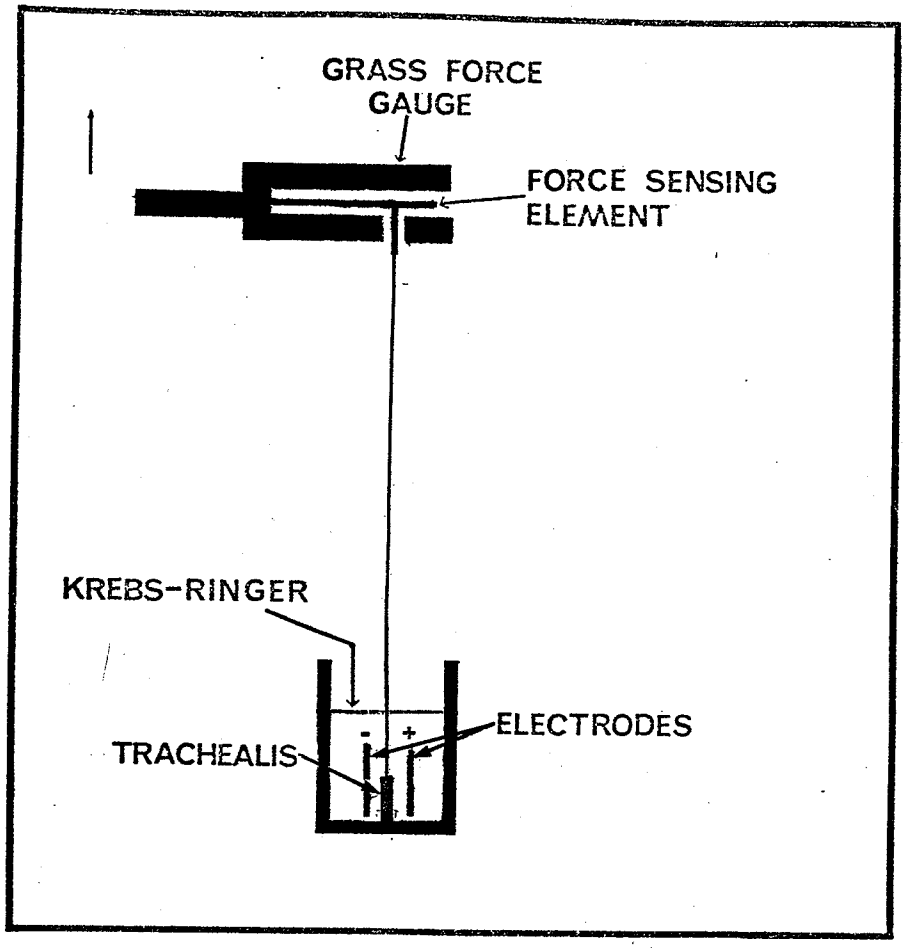

Figure 1: Schematic diagram of apparatus for mechanical studies (described in text).

the prepared strip was attached with a 000 braided surgical silk connection to a glass hook which was firmly clamped to the stand. The upper end was attached with similar material to a Grass FT .03 force transducer. This force transducer, through its rigid mounting on a Palmer stand could be moved vertically in the axis indicated by the arrow. The Palmer stand and bath were mounted on a heavy steel base plate to damp out extraneous vibrations. The output of the force transducer was recorded on a multi-channel Beckman dynograph recorder.

After mounting, the trachealis was allowed to incubate at 37° C for 2 hours to allow it to re-establish ionic equilibrium (Bohr, 1964). During this time it was stretched periodically (Speden, 1961) to facilitate equilibration.

Electrical field stimulation was effected by current from a 60 Hz source (Somlyo et. al., 1965) through platinum plate electrodes. The stimulus was of previously determined supramaximal field strength (400 mA/cm^2) and optimal duration. The resting and active length-tension relationship was then determined by stimulating the muscle electrically at five minute intervals between which the length was increased in measured increments. The resting tension (RP) just prior to stimulation and total tension (TP) elicited by stimulation were recorded, the active tension (AP) being the difference (TP-RP). The length at which active tension was maximal (L_{max}) was thus determined and that length maintained in subsequent experimental procedures.

C. STUDIES ON RISE OF RESTING TENSION

Since hypoxia had been observed to cause a spontaneous rise in resting tension (fig. 1a) the reversibility of the response was also observed by adding

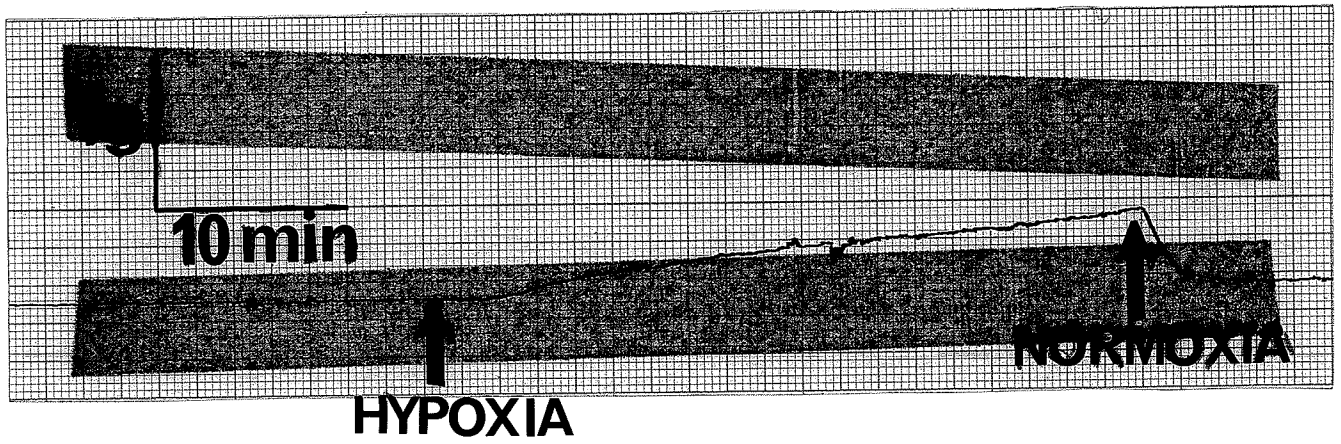

Figure 1a: Rise of resting tension in hypoxia (P_{O_2} < 10 mm Hg; dextrose-free) taken from a representative experiment. The muscle measured 0.9 cm x 0.15 cm x 0.08 cm and weighed 11 mg.

glucose and/or restoring the normal oxygen tension. The effects of 0.5 mM iodoacetate (IAA) and dinitrophenol (DNP) on resting tension were also observed.

D. ESTIMATION OF GLYCOGEN

I. Reagents

30% Aqueous potassium hydroxide solution (W/V)

Sulfuric acid. Reagent-grade 95.5% specific gravity 1.84.

80% phenol.80 grams redistilled reagent-grade phenol and 20 grams distilled water

Ethanol, absolute

Glycogen stock solution.25 mg purified glycogen from rabbit liver (Sigma) in 25 ml distilled water

II. Method

After the initial 2 hour equilibration period muscles at optimal length were subjected, in the succeeding hour to various concentrations of oxygen and dextrose with and without the presence of insulin (0.2 I.U./ml). Muscles were analyzed for glycogen content before and after the equilibration period as well as after the experimental period described above. The significance of observed differences was assessed by a one way analysis of variance and Duncan's multiple range test. The colorimetric determination employed was modified from that given by Montgomery (1957). Muscles were rapidly blotted, weighed and dropped into 2 ml of hot potassium hydroxide solution in a culture tube and the tubes placed in a boiling water bath until tissues were completely digested (about 45 minutes). The samples were then allowed

to cool to room temperature after which 0.050 ml saturated Na_2SO_4 solution and 2.0 ml absolute ethanol were added successively with vortex mixing of the sample after each addition. The samples were then placed in a hot water bath and heated to the point of gentle boiling for a period of 5-10 minutes. After cooling to room temperature the tubes were centrifuged and the supernatant discarded. Any remaining ethanol was evaporated by gentle heating. The precipitates were then dissolved in 2.0 ml distilled water and to all tubes, including suitable blanks was added 0.03 ml of phenol reagent. After thorough mixing, 3 ml concentrated sulfuric acid was added and the mixture vigorously shaken. The tubes were then allowed to stand for 60 minutes and the absorbance was recorded at 490 mμ as compared to the water blank. The absolute glycogen concentration was then determined from glycogen standards prepared from suitable dilutions of the stock solution. Recovery experiments were also conducted in which a known amount of glycogen was processed in parallel with the muscle digestion and precipitation procedures and the percent recovery calculated.

E. ESTIMATION OF HIGH ENERGY PHOSPHATES

I. Paper chromatography

a. Reagents

Perchloric acid 0.25 M

Norite suspension. 10 grams acid-washed Norite suspended in 90 grams distilled water.

Alcohol-ammonia reagent. 2 ml concentrated ammonium hydroxide and 60 ml absolute ethanol diluted with water to a final volume of 100 ml.

Isobutyric acid-ammonium isobutyrate solvent. 125 ml isobutyric acid brought to pH 3.6 - 3.7 by the addition of about 75 ml 0.5 N ammonium hydroxide

b. Method

1. Extraction

Muscles were extracted, after equilibration at optimal length, by a modification of the method of Zymaris et. al. (1959). Muscles were rapidly frozen in liquid nitrogen, weighed, and ground in a liquid nitrogen-pre-cooled mortar and pestle with 10 ml perchloric acid per gram tissue. The mixture was then thawed and centrifuged at 3000 RPM for 15 minutes at 0° C. The Norite was then centrifuged out, washed with distilled water and re-centrifuged. Alcohol-ammonia reagent (8 ml/gm muscle) was then added and elution carried out overnight at room temperature. The Norite was then centrifuged out and the supernatant concentrated to a small measured volume.

2. Resolution

A total of 0.03 ml of this solution was then applied, in a number of small serial applications to sheets of Whatman #1 chromatography paper measuring 18 1/2 x 22 1/4 inches. Known standard solutions of ATP, ADP and AMP were applied to each sheet to serve as reference markers for these substances. The mixtures were then resolved by ascending chromatography for 24 hours using the isobutyric acid-ammonium isobutyrate solvent system after which the solvent front was marked and the chromatogram dried. The resolved nucleotides were visualized under short wavelength ultraviolet light and outlined. The geometrical centers of the respective spots were marked

and R_f values were calculated.

3. Quantitative estimation

The outlined nucleotides were eluted with 0.1 N HCl and the optical density (O.D.) values determined at 257 and 300 mμ. Appropriate reagent and paper blanks as well as standard reference spots were determined for each nucleotide. Thus the amount of each nucleotide (unk) in each tissue could be determined from the equation:

μmoles nucleotide/gm tissue =

$$\frac{(\text{O.D. unk}_{257} - \text{O.D. unk}_{300})\,(\text{O.D. blank}_{257} - \text{O.D. blank}_{300})}{(\text{O.D. Std}_{257} - \text{O.D. Std}_{300})\,(\text{O.D. blank}_{257} - \text{O.D. blank}_{300})} \times \frac{\text{μmoles Std. nucleotide}}{\text{wet weight of tissue (gm)}}$$

II. Enzymatic determinations

a. ATP-CP

1. Principle of the method

The reactions:

(1) 3-phosphoglyceric acid + ATP $\xrightarrow[(3\text{-PGK})]{\text{3-phosphoglyceric kinase}}$ 1,3 diphosphoglyceric acid + ADP

(2) 1,3 diphosphoglyceric acid + NADH + H^+ $\xrightarrow[(\text{GAPDH})]{\text{glyceraldehyde phosphate dehydrogenase}}$ glyceraldehyde phosphate + NAD^+ + H_3PO_4

can be coupled to measure limiting amounts of ATP through the decrease in optical density of NADH at 340 mμ as it is consumed in the reactions outlined. The subsequent determination of creatine phosphate can be made by the addition of creatine phosphokinase (CPK) which catalyzes the reaction:

(3) Creatine phosphate + ADP $\xrightarrow{CPK}$ creatine + ATP

The ATP so formed is immediately measured through reactions (1) and (2) above and, since, all reactions proceed stoichiometrically and the molar extinction coefficient for NADH is known, the amount of ATP and CP initially in the extract can be calculated.

2. Reagents

Tra-EDTA buffer pH 7.6. 0.1 M triethanolamine and 0.01 M disodium ethylenediamine tetracetate. The pH was adjusted to 7.6 with HCl.

Tra-KCl buffer pH 7.5. 0.33 M triethanolamine and 1.1 N KOH brought to pH 7.5 with HCl

0.5 M perchloric acid

0.5 M $MgSO_4$

0.1 M 3-phosphoglyceric acid, tricyclohexylammonium salt. $3H_2O$ (PGA) (Boehringer)

0.1 M NADH (Sigma)

0.05 M reduced glutathione (GSH)(Boehringer)

0.01 M ADP $Na_3 \cdot H_2O$ (Boehringer)

All reagents were dissolved in deionized glass redistilled water except for NADH which was dissolved in Tra-EDTA buffer and freshly prepared for each experiment.

The enzymes creatine phosphokinase (CPK), 3-phosphoglyceric kinase (PGK) (2 ml protein/ml) and glyceraldehyde phosphate dehydrogenase (GAPDH) (10 mg protein/ml) were obtained from the Boehringer Mannheim Corp., New York. CPK was obtained as a lyophilized powder and was suspended in 2M ammonium sulphate solution (20 mg/ml).

3. Method

Muscles, after the 2 hour equilibration period in dextrose-free medium at optimal length were divided into two groups, the control group receiving normal oxygen concentrations in the succeeding hour and the experimental group hypoxia (P_{O_2}<10 mm Hg). P_{CO_2} and pH were held constant. The muscles were also stimulated regularly at 5 minute intervals electrically, and developed tension was monitored to confirm the mechanical condition of the muscles.

The extraction of nucleotides from muscle was modified from that described by Cain and Davies (1962). Muscles were rapidly frozen by plunging them into liquid nitrogen five minutes after the last stimulation. Any frozen Ringer's solution adhering to the muscle surface was then carefully scraped off and the muscle weighed, care being taken to ensure that the muscle remained near the temperature of liquid nitrogen (B. P. -196° C.) throughout the process. The muscles were then transferred to pre-cooled stainless steel centrifuge tubes and pulverized to a smooth powder in the presence of 0.3ml of 0.5 M perchloric acid with a loosely fitting similarly pre-cooled magnesium rod. Extracts were stored in liquid nitrogen. They were then thawed in pairs, 1.0 ml 0.5 M perchloric acid was added and the mixture incubated at 35° C. for four minutes with stirring. They were immediately centrifuged (3000 RPM x 2 min) and 1.0 ml of the supernatant rapidly removed and added

to 0.45 ml of Tra-KCl buffer pH 7.5. The solutions were then refrozen and stored in liquid nitrogen until ready for analysis. The maximum storage time was 2 hours, analyses being done immediately after extraction of all the tissues to avoid decomposition of the high energy compounds due to longer periods in storage. Upon rethawing the $KClO_4$ precipitate was centrifuged out at 0° C and the supernatant neutralized with 30% KOH (70 µl).

The method for the estimation of ATP and CP was modified from the micro-method described by Fawaz et. al. (1962). Into a semi-micro cuvette (light path 1 cm, width 0.4 cm) of a Hitachi Perkin-Elmer UV-Vis model 139 spectrophotometer the following reagents were pipetted (in order):

0.340 ml Tra-EDTA

0.015 ml $MgSO_4$

0.012 ml DPNH

0.70 ml 3-PGA

0.035 ml GSH

0.010 ml ADP

0.500 ml sample

Three readings of the optical density at 340 mµ were taken at 2 minute intervals after stirring with a plastic plunger. The enzymes for the estimation of ATP were then pipetted on the foot of the plunger: 3 µl GAPDH and 5 µl PGK and introduced to the cuvette with mixing. Readings were continued at 2 minute intervals until no further decreases in optical density occurred or the rate of decrease was slow and constant. The enzyme CPK (10 µl) was

then similarly added with mixing and the readings continued until termination of the reaction. The total decrease in optical density (ΔE) in the respective reactions was noted.

4. Calculations

Since the reactions proceed stoichiometrically, the amounts of ATP and CP originally in the muscle extract were calculated from the decrease in optical density (ΔE) and the molar extinction coefficient of NADH (6.22). Appropriate standards were also measured as a check on the method. The equation applicable for this procedure and the particular dilutions employed was:

$$(4)\quad \frac{\mu\text{moles ATP (or CP)}}{\text{g wet weight}} = \frac{\Delta E}{6.22} \times \frac{\text{Tissue wt. (mg)}}{1.3 + \text{tissue wt (g)}} \times \frac{0.5}{1.52}$$

b. ADP-AMP

1. Principle of the method

When the amount of ADP present is limiting, the reactions

$$(5)\quad \text{ADP + phosphoenol - pyruvic acid} \xrightarrow[(PK)]{\text{pyruvate kinase}} \text{ATP + pyruvate}$$

and

$$(6)\quad \text{pyruvate + NADH + } H^{+} \xrightarrow[(LDH)]{\text{lactic dehydrogenase}} \text{lactate + } NAD^{+}$$

can be utilized to measure ADP through the decrease in optical density (ΔE) at 340 mμ due to NADH as it is consumed. After the completion of these reactions, AMP can be determined in the same assay mixture since AMP can

be converted to ADP in the myokinase (MK) catalyzed reaction:

(7) $$AMP + ATP \xrightarrow{MK} 2\ ADP$$

and the resulting ADP determined through reactions (5) and (6). In this determination, however, it is noted that one AMP gives rise to two ADP and this fact must be taken into account in calculations.

2. Reagents

Tra-EDTA buffer, pH 7.6. 0.1 M triethanolamine and 0.01 M disodium ethylenediamine tetraacetate. The pH was adjusted to 7.6 with HCl.

PEP/Mg^{++}/K^{+} solution. 0.01 M phospho-enol-pyruvate, cyclohexyl-ammonium salt, (Boehringer) 1.3 M KCl, 0.4 M $MgSO_4$

0.01 M NADH (Sigma)

All reagents were dissolved in deionized glass redistilled water except for NADH which was dissolved in Tra-EDTA buffer and freshly prepared for each experiment.

The enzymes lactic dehydrogenase (LDH), (1 mg protein/ml), pyruvate kinase (PK) (1 mg protein/ml) and myokinase (MK) (2 mg protein/ml) were obtained from the Boehringer Mannheim Corp., New York.

3. Method

The muscle extracts described above were analyzed not only for ATP and CP content but also for ADP and AMP content. The method used for the estimation of ADP and AMP was modified from that described by Boehringer in its ADP/AMP combination test kit. Into a semi-micro cuvette (light path 1 cm, with 0.4 cm) of a Hitachi Perkin-Elmer UV-Vis model 139 spectrophotometer the following reagents were pipetted in this order:

0.400 ml Tra-EDTA buffer

0.070 ml PEP/Mg^{++}/K^{+}

0.010 ml NADH

0.010 ml LDH

0.500 ml sample

After stirring with a plastic plunger, three readings of optical density at 340 mμ were made at 2 minute intervals. The enzyme pyruvate kinase was then pipetted (10 μl) on the foot of the plunger and added to the solution with stirring. Readings of optical density were continued at 1 minute intervals thereafter until the reactions stopped. The AMP content was then determined by similarly adding 10 μl of myokinase and continuing the readings of optical density until termination of the reaction. The total decrease in optical density (Δ E) in the respective reactions was noted.

4. Calculations

It was noted that the reactions for the determination of ADP proceed stoichiometrically with one NADH consumed per ADP converted to ATP while in the determination of AMP two NADH are consumed per AMP converted to ADP. Thus the equation given for the calculation of tissue ATP and CP contents is applicable for the calculation of ADP and AMP as well with the modification that in the calculation of AMP the value obtained should be multiplied by 0.5. The significance of differences in mean levels of the respective compounds was assessed by the student's t-test.

F. ESTIMATION OF TISSUE CALCIUM CONTENT

I. Principle of the method

The physical principle underlying atomic absorption spectrophotometry is the observation that the light emitted by the glowing vapour of an element is a discontinuous or line spectrum characteristic of that element and that light passing through a vapour of that same element is absorbed at the same frequencies as are emitted by the glowing vapour. The absorbance, moreover, is related to the concentration of the vapour. In the atomic absorption spectrophotometer a calcium vapour lamp emits the line spectrum characteristic of calcium and this spectrum is passed through a flame in which is being vapourized a solution containing calcium. The intensity of one of the emitted spectral lines, selected with a monochromator, is monitored by a photomultiplier tube and is found to vary inversely with the amount of calcium in solution.

II. Reagents

0.5 N NaOH

3.75 % Na_2EDTA

All glassware was carefully cleaned of calcium contamination by sonication in distilled water containing EDTA and rinsed with deionized, glass redistilled water.

III. Method

After 2 hours dextrose-and phosphate-free equilibration at optimal length paired muscles were equilibrated in the succeeding hour with 1) normal oxygen

and calcium concentrations (control) 2) hypoxia ($P_{O_2} < 10$ mm Hg) with normal external calcium concentrations 3) normoxia but in Ca^{++}-free Krebs-Ringers containing 5 mM Na_2EDTA and 4) hypoxia and Ca^{++}-free Krebs-Ringers containing 5 mM Na_2EDTA. The muscles were then dipped momentarily into a Ca^{++}-free Krebs-Ringer solution to remove any external calcium adhering to the muscle and the wet weight measured after drawing along clean waxed paper to remove any adhering solution (Bauer et. al., 1965). They were then dried in an oven at 110° C overnight and the dry weights recorded. Digestion of the muscles was accomplished by incubating them overnight in 2 ml 0.5 N NaOH at 37° C. To these solutions was added 20 µl 3.75% EDTA solution and deionized glass redistilled water was then added to a final volume of 10 ml. This mixture was then Millipore filtered (Metricel, pore size 0.45 µ) to remove any remaining particulate matter. Appropriate standards, made up by serial dilution of a stock 1 mM $CaCO_3$ solution, and blanks were analyzed with the muscle extracts.

Analysis for the calcium content of these solutions was carried out on a Perkin Elmer model 303 dual beam atomic absorption spectrophotometer. After adjusting the flame to a pale white and optimizing meter response at 210.6 mµ the absorbance was read for the various standards, blanks and samples and the calcium content of the samples calculated from the standard curve after correcting for blank readings.

G. MEASUREMENT OF CALCIUM^{45} FLUXES

I. Reagents

Bray's (1960) solution. 60 g naphthalene, 4 gm 2,5-diphenyloxazole (PPO), 0.2 gm 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (dimethyl POPOP), 100 ml methanol, 20 ml ethylene glycol and p-dioxane to a total volume of 1 liter

$Ca^{45}Cl_2$ solution. 1 mC $Ca^{45}Cl_2$ in 0.1 ml 0.1 N HCl (New England Nuclear)

II. Method

After the initial 2 hour equilibration period muscles at optimal length were equilibrated a further 2 hours in dextrose-free phosphate-free Krebs-Ringer solution containing 7 μC/ml Ca^{45}. A control group was normally oxygenated and experimental muscles were made hypoxic ($P_{O_2} < 10$ mm Hg) during this loading period. Following this loading period muscles were momentarily dipped in two successive non-radioactive solutions to remove adhering radioactivity and placed in a vial containing 10 ml non-radioactive solution which was rapidly mixed through vigorous bubbling of the gas mixture through the solution. Samples (100 μl) of the solution were withdrawn at measured time intervals in the succeeding 140 minutes and the radioactivity of these samples measured. This was done by placing them in vials containing 15 ml Bray's solution and counting them in a Packard Tri Carb model 3375 liquid scintillation spectrometer with appropriate correction for quenching and volume lost due to the withdrawal of previous samples. Control muscles were occasionally made hypoxic at the 140 minute mark and samples withdrawn

for a further 20 minutes while tension was recorded simultaneously. The net Ca^{45} efflux was then plotted semilogarithmically against time, an eye line of best fit applied to the data points, and the curve analyzed for its component exponentials by the graphical method of Riggs (1963). The significance of differences of mean compartment half times was assessed by student's t-test.

H. EFFECT OF ACETYLCHOLINE ON Ca^{++}-FREE MUSCLES

The effectiveness of acetylcholine in repeatedly stimulating the muscle in a calcium-free (0.1 mM EDTA) medium was tested after electrical stimulation and potassium depolarizing solutions were no longer effective.

RESULTS

A. MECHANICAL STUDIES

I. Length-tension relationship

The length-tension relationship of airway smooth muscle has been reported previously (Stephens et. al., 1968; Stephens et. al., 1969) and the results of a typical experiment on the trachealis are shown in figure 2. Muscle length (in mm) is plotted against the tension (in grams) developed by the muscle at rest (RP) and under supramaximal tetanic stimulation (TP). The difference between these two (AP) is also plotted. The length at which active tension is maximal is termed the optimal length or L_{max} of the tissue and this length was maintained in all subsequent studies. It will be noted that the Frank-Starling length dependency of active tension characteristic of striated and cardiac muscle is also characteristic of this muscle; and also that the RP at L_{max} is low relative to AP_{max} ($RP \approx 5\%\ AP_{max}$).

II. Reversibility of RP rise and effect of inhibitors

In investigating the reversibility of the rise in tension by the subsequent addition of dextrose or normal oxygen concentrations it was found that the phenomenon was rapidly reversible with resting tension returning to normal within three minutes of these additions.

The metabolic inhibitors IAA and DNP, like hypoxia (Stephens and Kroeger, 1970) caused a rapid decline in active muscle function and an increase in resting tension.

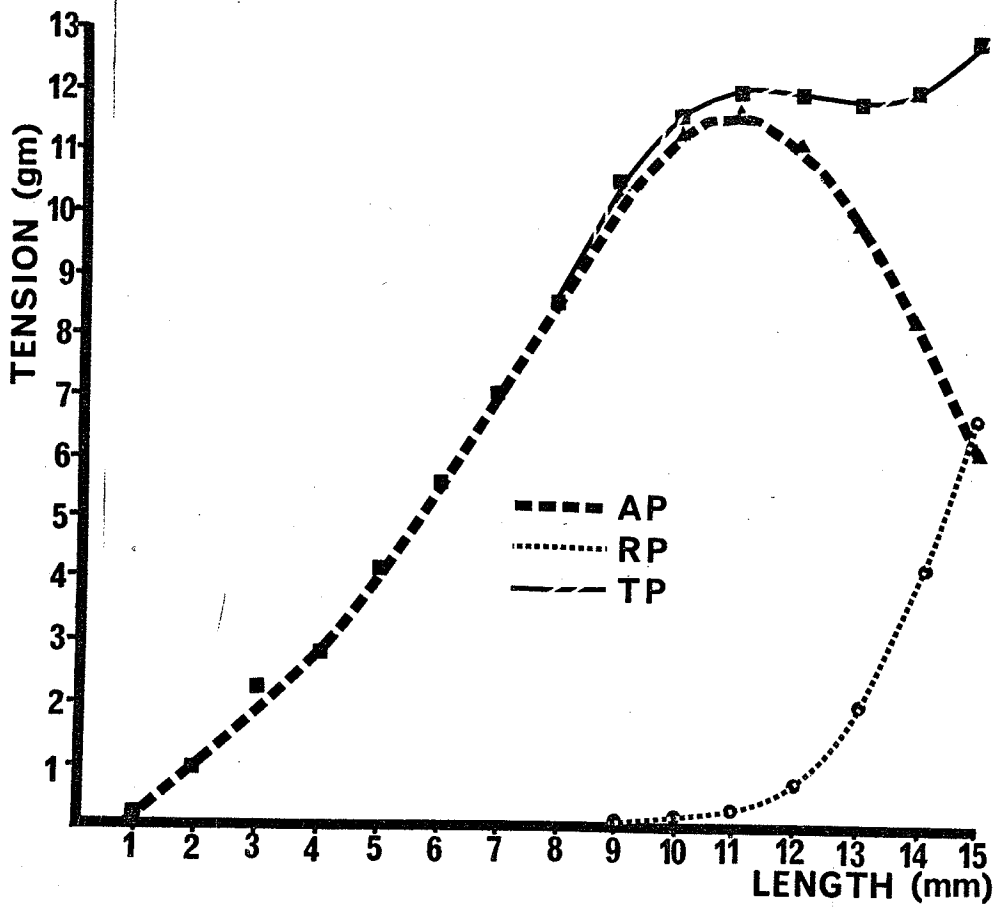

Figure 2: Length-tension relationship in the trachealis (taken from a representative experiment). The resting (RP), active (AP) and total (TP) tension curves are shown. Note the low resting tension at the length where active tension is maximal. The wet tissue weight was 11 mg.

B. GLYCOGEN CONTENT OF TISSUES

Recovery experiments indicated that the recovery of glycogen by this method was essentially complete. The glycogen content of the muscles after the various equilibration conditions is shown in figure 3 and table 2. A slow, linear decrease in muscle glycogen in the presence of dextrose and oxygen was noted. The rate of glycogenolysis calculated from the decrease in mean glycogen content as a function of time was 0.27 mg/g tissue/hr. Glycogenolysis similarly calculated was somewhat more rapid (0.44 mg/g tissue/hr) in the absence of external dextrose though the difference was not statistically significant. Hypoxia, however, was a much more potent stimulus to glycogenolysis, the rate being 1.87 mg/g tissue/hr with dextrose present and 2.5 mg/g/hr in the dextrose-free muscles in spite of the fact that the muscles were not stimulated in the experimental period. These values were significantly different ($P<0.05$) from control values and from each other as determined by a one-way analysis of variance with the significance of differences assessed by Duncan's multiple range test. Thus the rates of glycogenolysis were increased by factors of 6.9 and 5.7 respectively over their resting values. Of special interest was the observation that the presence of insulin with dextrose and oxygen did not inhibit glycogenolysis and that, at the same time, it appeared to protect partially the glycogen stores from rapid glycogenolysis in the presence of hypoxia.

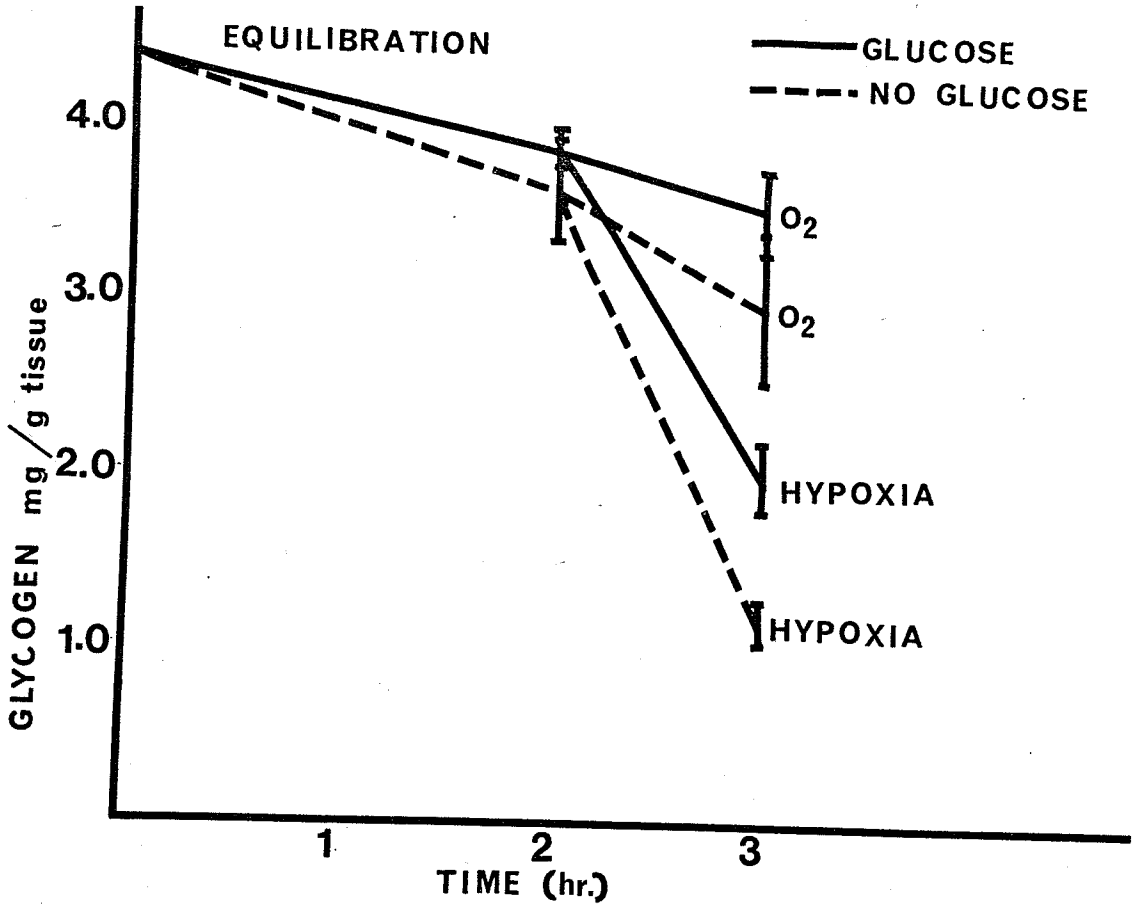

Figure 3: Effect of hypoxia and substrate depletion on trachealis glycogen content. Vertical bars represent S.E.

TABLE 2

Effect of Hypoxia, Dextrose and Insulin on Trachealis Glycogen Stores

Time	Equilibration conditions	Determinations (N)	Glycogen content (mg/g tissue) $\bar{X} \pm$ S.E.
t = 0	--	12	4.34 ± 0.40
t = 2 hr	Dextrose, oxygen	23	3.86 ± 0.27
	Dextrose-free, oxygen	17	3.67 ± 0.32
t = 3 hr	Dextrose, oxygen	6	3.56 ± 0.48
	Dextrose, oxygen, insulin (1 hr)	5	3.50 ± 0.54
	Dextrose, hypoxia (1 hr)	7	1.99 ± 0.21
	Dextrose, hypoxia, insulin (1 hr)	5	3.13 ± 0.36
	Dextrose-free, oxygen	7	2.98 ± 0.43
	Dextrose-free, hypoxia (1 hr)	11	1.17 ± 0.12

C. HIGH ENERGY PHOSPHATE CONTENTS

I. Paper chromatography

A typical chromatogram of eluted nucleotide phosphates and standards is presented in figure 4. Clear separation of the nucleotides ATP, ADP and AMP was obtained in the chromatogram of the standard mixture (B). Spots corresponding in R_f value (calculated as: distance of spot from origin/ distance of solvent front from origin) to those found for the standard mixture were taken as preliminary evidence of the separation of ATP, ADP and AMP extracted from muscle. However it was not possible to discern the presence of other nucleotides such as GTP and GDP on the chromatogram. Presumably they were present in quantities which were small in comparison to the adenosine phosphates and could not be detected by this method. This finding was important to the interpretation of the enzymatic determinations of ATP and ADP since the enzymes phosphoglycerate kinase and pyruvate kinase are not specific for ATP and ADP respectively and the presence of large amounts of, for example, GTP and GDP would interfere with the determination. Although the spots corresponding to those of ATP, ADP and AMP were readily distinguishable, quantitative estimation of these compounds by subsequent elution and absorbance readings was not successful because of insufficient resolution of the spectrophotometer to detect the small quantities present.

II. Enzymatic determinations

Typical absorbance changes with time in the coupled enzymatic determination of ATP/CP and ADP/AMP are shown in figure 5 a and b respectively.

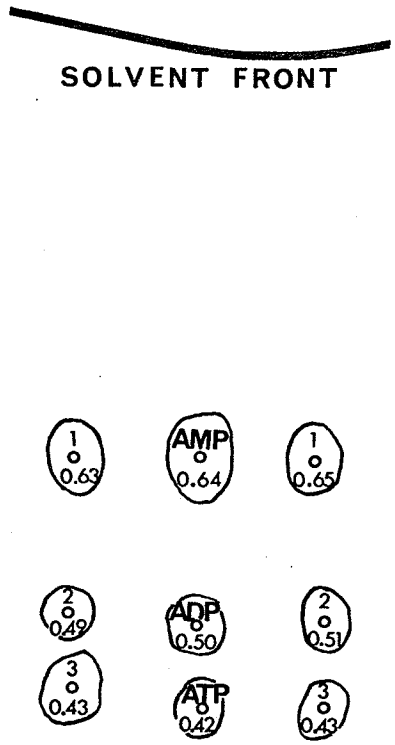

Figure 4: Chromatogram of nucleotide phosphates in the trachealis (taken from an experiment representative of the six performed). Extracts of two muscles (A and C) were resolved simultaneously with a standard solution (B) containing AMP, ADP and ATP. The spots are numbered 1 through 3 in the order of their R_f values. The geometrical centers of the spots are indicated by ∘ and the calculated R_f values are given in the lower half of each spot. Note the similarity of the pattern of spots resolved from the muscle extracts with that of the standard solution.

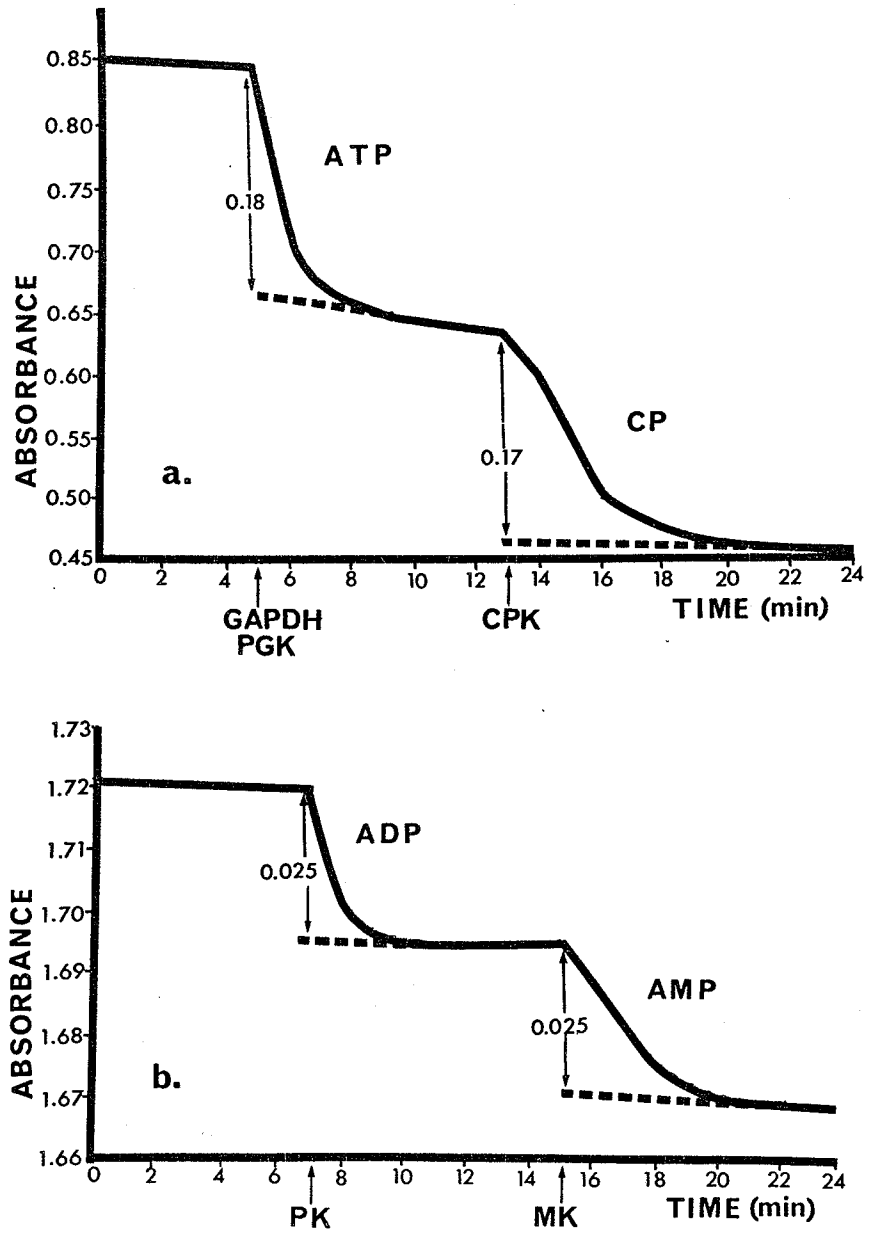

Figure 5: Absorbance changes in the course of the determination of a) ATP/CP and b) ADP/AMP in muscle extracts, taken from a representative experiment. Arrows indicate the addition of the indicated enzymes and start of the reactions. Since some enzymatic reactions do not stop completely, a slow continuous decrease in optical density ("creep") is noted. This is indicated by a small slope in the extrapolation line (broken). Thus the Δ E value at the time of addition of the enzyme (values given in figure) was calculated. The second enzyme in the respective coupled systems was added after the change in optical density of the first reaction had become a linear function of time.

Determinations of standard nucleotide solutions indicated that the accuracy of the method was 95% or greater.

The tissue concentrations of CP, ATP , ADP and AMP under control and hypoxic conditions are shown in figure 6. In hypoxia, CP decreased from $0.935 \pm .069$ to 0.160 ± 0.036 μmoles/gram and ATP decreased from 1.119 ± 0.042 to 0.681 ± 0.089 μmoles/gram ($P < 0.05$). The small mean increase in ADP content was not statistically significant. The increase of AMP content from 0.048 ± 0.037 to 0.205 ± 0.037 μmoles/gram however, was significant ($P < 0.05$).

D. TISSUE CALCIUM CONTENTS

The total calcium content of tissues equilibrated with various concentrations of oxygen and Ca^{++} are shown in figure 7. The normal trachealis calcium content in the presence of 1.91 mM external Ca^{++} was 1.60 ± 0.12 mmoles/kg wet weight and increased to 1.80 ± 0.12 mmoles/kg after one hour exposure to hypoxia. The mean calcium content of muscles equilibrated for one hour with Ca^{++}-free Ringer containing EDTA in the presence of normal oxygen concentrations was 0.91 ± 0.05 mmoles/kg while in the presence of hypoxia, the mean calcium content decreased to 0.71 ± 0.04 mmoles/kg wet weight. Both of these control-hypoxia differences between paired muscles were shown to be statistically significant ($P < 0.05$) by a one-way analysis of variance with the significance of differences assessed by Duncan's multiple range test.

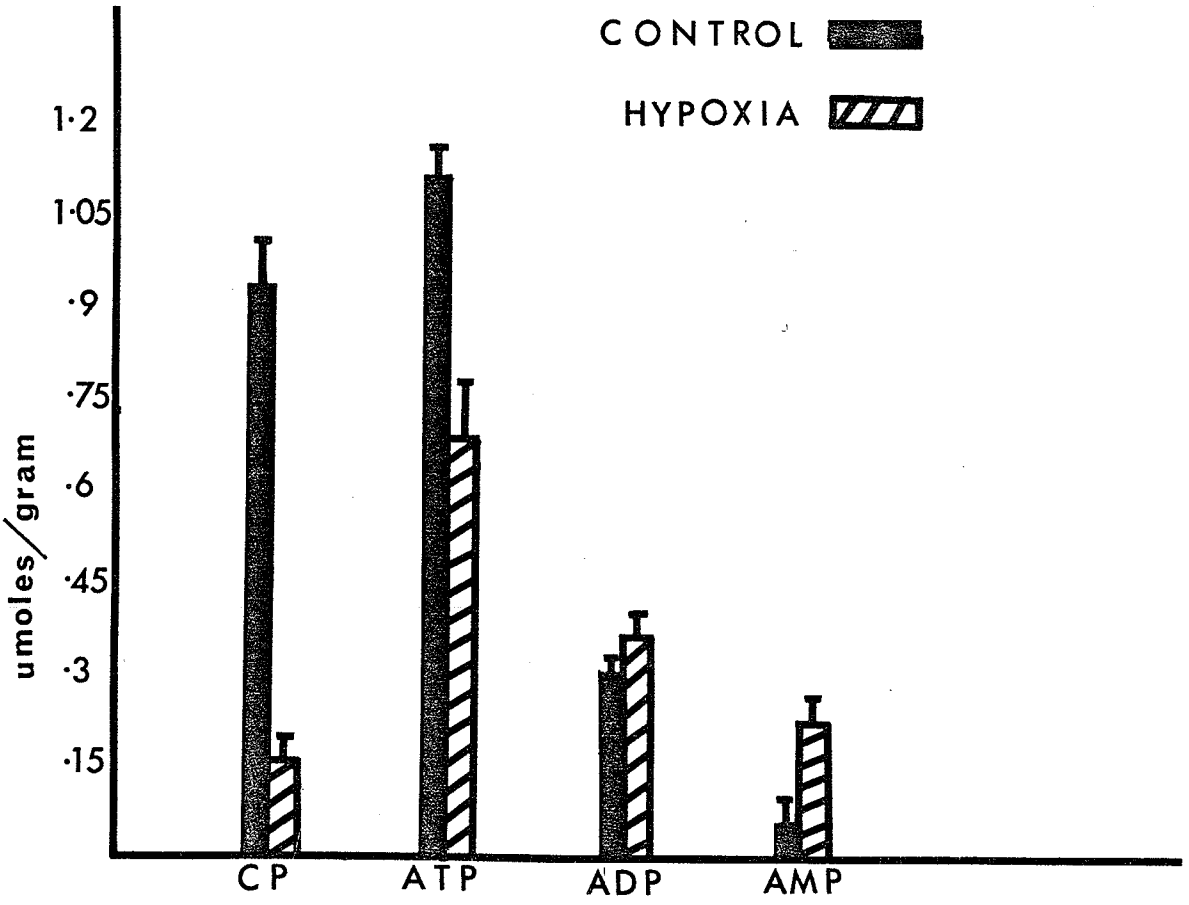

Figure 6: High energy phosphate stores in the trachealis. The values graphed represent the means and S. E. (small vertical bars) of measurements on twelve muscles. All muscles were dextrose-free . (Hypoxia = P_{O_2} < 10 mm Hg for 1 hour - see text).

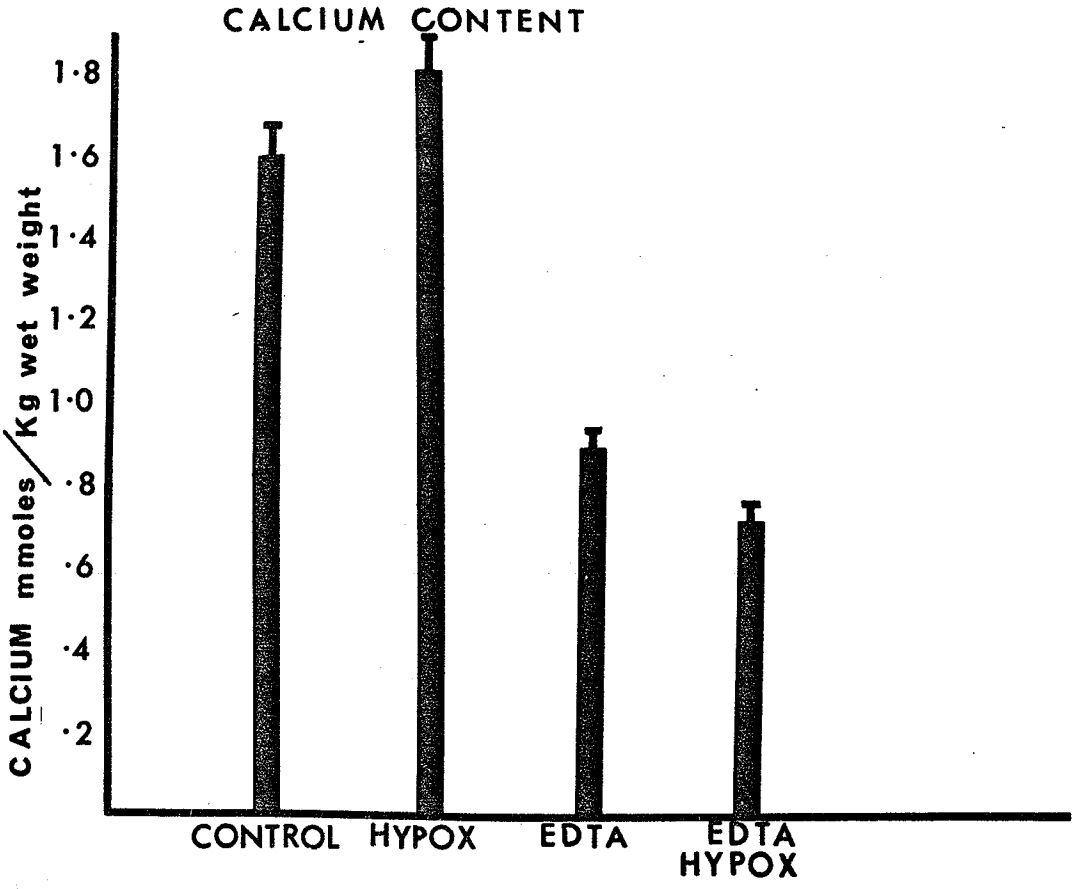

Figure 7: Effect of hypoxia on tissue calcium levels in the trachealis. The values graphed represent the means and S.E. of analyses of the following numbers of muscles: control - 12, hypoxia - 10, EDTA - 10, and EDTA hypoxia - 17 after one hour under experimental conditions. See text for further details.

E. CALCIUM^{45} EFFLUXES

The data points, fitted by an eye line and graphical analysis of its component exponentials and equation of a typical Ca^{45} efflux from trachealis smooth muscle are shown in figure 8. It will be noted that at least three exponentials can be resolved in the efflux curve: one (line C) with a half time ($t_{1/2}$) of 0.65 minutes, another (line B) with a $t_{1/2}$ of 11.5 minutes and a slowly exchanging exponential with a $t_{1/2}$ of 603 minutes. Analysis of 10 paired (hypoxic-normoxic) efflux curves showed (table 3) that the mean half times of the respective exponentials was not significantly changed in hypoxia. However, if the muscles were made hypoxic at a time when Ca^{45} efflux had become low and fairly constant, Ca^{45} efflux was observed to increase at the same time that tension began to rise as is shown in figure 9.

F. EFFECT OF ACETYLCHOLINE (ACh) ON Ca^{++}-FREE MUSCLES

The effect of a submaximal dose of ACh (10^{-5} g/ml.) on muscle in Ca^{++}-free Ringers solution for a sufficient length of time to abolish contractile responses to electrical stimulation and potassium was to cause a phasic contraction (in contrast to its tonic response in Ca^{++}-containing Ringers) with a small amount of tension being sustained for a long period of time (figure 10). It was of interest to observe that, if the ACh was subsequently washed out while Ca^{++}-free conditions were maintained, another ACh stimulus applied a short time later caused another phasic contraction whose peak exceeded the trough of the previous contraction. The effect of ACh was abolished in severe hypoxia, however.

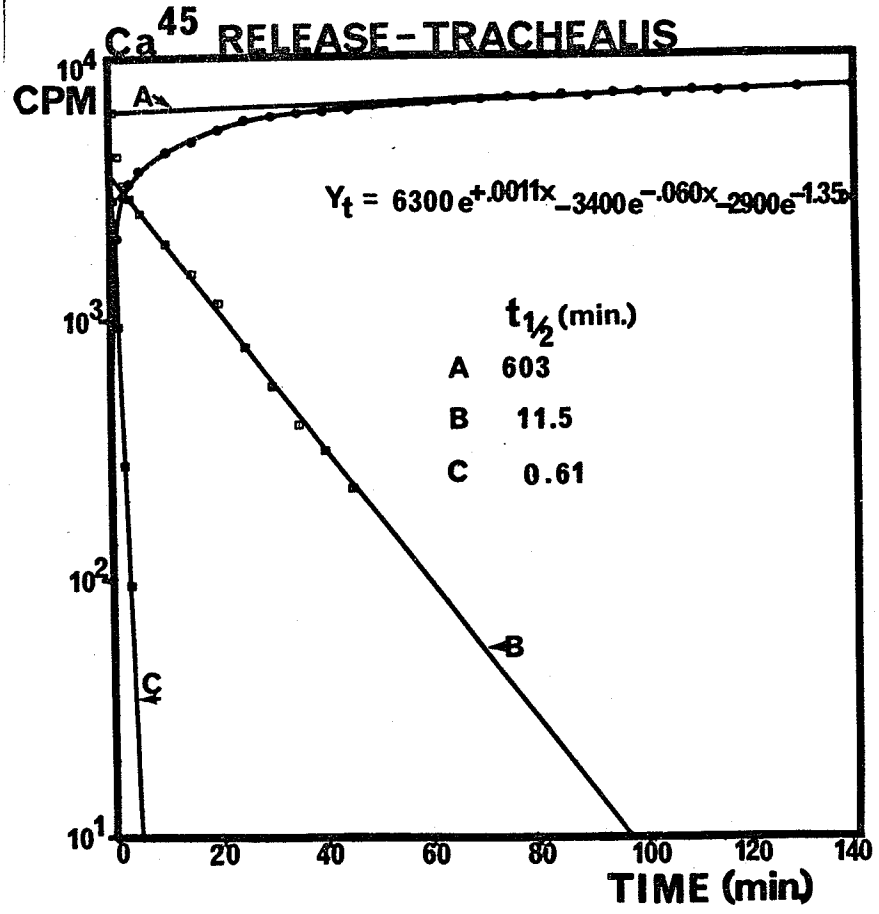

Figure 8: Efflux of Ca^{45} from the trachealis showing the graphical method employed in solving for the respective exponentials. A curve has been fitted by eye to the data points (closed circles). Line A is an extrapolation of the linear portion of the data curve. The open squares to which line B has been fitted represent the deviation of the data curve from line A in the time period up to 50 minutes. The closed squares to which line C has been fitted similarly represent the deviation of the first three open squares from line B. The slopes of the respective lines were converted mathematically to the half times shown.

TABLE 3

Effect of Hypoxia on Ca^{45} Kinetics in Trachealis

	Compartment half times (min)		
	Fast	Medium	Slow
Normoxia	0.72 ± 0.18	12.22 ± 1.35	471.0 ± 42.2
Hypoxia	0.88 ± 0.13	11.86 ± 1.00	530.6 ± 56.1

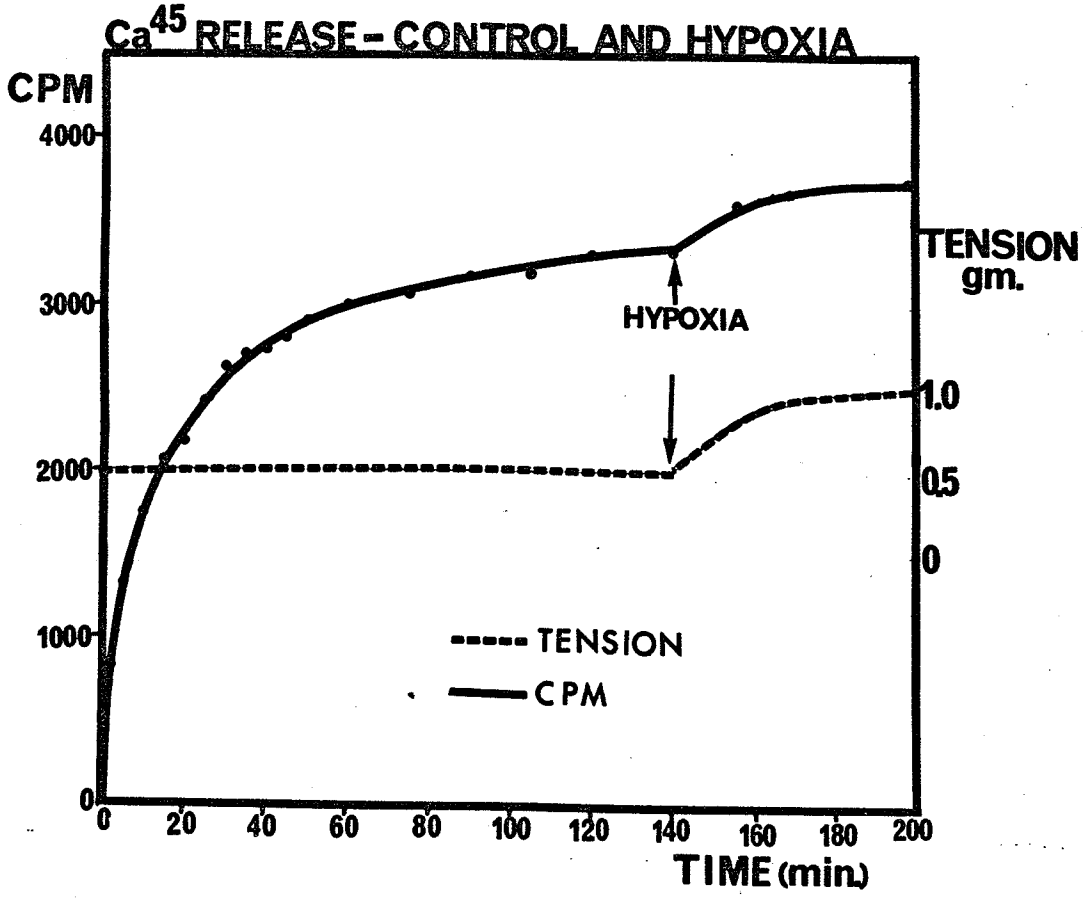

Figure 9: Effect of hypoxia on Ca^{45} efflux and resting tension, taken from a representative experiment. The increased Ca^{45} efflux in hypoxia was noted in five experiments. See text for details.

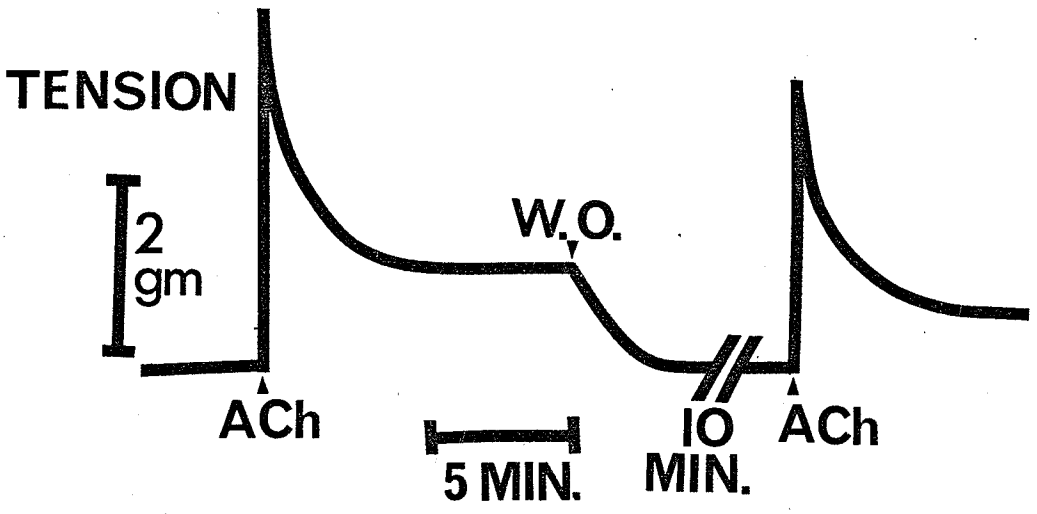

Figure 10: Effect of Ca^{++}-free equilibration on trachealis responses to 10^{-5} g/ml acetylcholine (ACh). See text for details.

DISCUSSION

A. MECHANICAL STUDIES

Although active responses of smooth muscle are inhibited by hypoxia and metabolic inhibitors in most smooth muscles (Bülbring and Lüllmann, 1957; Lloyd, 1967; Coe et.al.,1968; Detar and Bohr, 1968 a; Marshall, 1962) an increase in resting tension at the same time, similar in some respects to rigor in skeletal muscle, has not been observed in smooth muscle other than the trachealis (Stephens and Kroeger, 1970). A possible exception is pulmonary vascular smooth muscle (Lloyd, 1967, 1970) but this effect may not be directly a consequence of hypoxia on the smooth muscle (Lloyd, 1968). An analog of this phenomenon is the decreased compliance and increased airway resistance reported by Tisi et.al. (1970) for hypoxic canine lungs. The observations that the effects of hypoxia could be duplicated by the addition of metabolic inhibitors and that they could be rapidly reversed by the addition of oxygen and/or dextrose suggested that the role of hypoxia in the observed mechanical changes was its inhibition of metabolism supplying energy for active processes such as active tension development, and, perhaps, the maintenance of a high pCa intracellularly. The experiments discussed below were designed to test various predictions of this hypothesis.

B. EFFECT OF HYPOXIA ON GLYCOGEN CONTENT

A number of factors such as dietary state, exercise, hormonal state, acidosis and anoxia affect muscle glycogen stores (West et.al.,1966).

In view of the varying dietary history, stress of handling and tissue hypoxia in anaesthesia of mongrel dogs, a wide scatter of individual absolute values was inevitable. However, the pattern of values found in any given experiment conformed to the pattern of mean values shown in figure 3. The normal glycogen levels found in the trachealis are comparable to those reported for other muscles and a comparison is made in table 4. In the presence of control oxygen concentrations glycogenolysis was slow (0.27 to 0.44 mg/g wet tissue/hr) and is similar to data reported for taenia coli (Bueding and Bülbring, 1964). In contrast, glycogenolysis in the rat uterus under identical conditions is much more rapid, being 0.8 mg/g/hr in the uteri of ovariectomized rats and up to 7.1 mg/g/hr in the uteri of estradiol and progesterone treated rats (West and Cervoni, 1955). Since carbohydrates are a major source of energy in smooth muscle (Somlyo and Somlyo, 1968; Needham and Shoenberg, 1967) an increase in glycogenolysis in dextrose-free hypoxic equilibration conditions was expected and, in fact, the rate reached at least 2.5 mg/g/hr though the finding that glycogenolysis could be increased 5-7-fold by hypoxia contrasts with the findings for vascular muscle (Kirk et. al., 1954) and indicates that the trachealis relies to a much greater extent on oxidative metabolic pathways. It is important to note however, that other sources of energy such as lipids and the HMP shunt may be quantitatively important in aerobic muscle (evidence reviewed in introduction). At the end of this period of hypoxia, although the contractile function of trachealis was almost completely abolished, a significant amount of glycogen

TABLE 4

Glycogen Stores in Muscles

Tissue	Glycogen (mg/g)	Reference
guinea pig skeletal muscle	9.0	Montgomery, 1957
rat heart	4.97	Daw, et.al., 1968
rat intestine	1.14	Lundholm and Mohme-Lundholm, 1963
bovine trachealis	2.51	Lundholm and Mohme-Lundholm, 1963
bovine mesenteric artery	1.61	Lundholm and Mohme-Lundholm, 1963
rat uterus (ovariectomized)	0.43	West and Cervoni , 1955
rat uterus (ovariectomized with estradiol and progesterone)	2.07	West and Cervoni, 1955
canine trachealis	4.34	(present study)

remained which appeared to be sequestered since it could not be depleted and the mechanical effect of hypoxia at this point was irreversible.

While these observations are consistent with those in most tissues, the differences in the effect of a lack of external dextrose on mechanical activity, glycogen stores and high energy phosphates in taenia coli and the trachealis are interesting. Bueding et. al. (1967) have reported that after one hour equilibration in a dextrose-free, oxygenated medium, the active and baseline tension of taenia coli fell to very low levels and high energy phosphates were markedly reduced. Axelsson and Bülbring (1959) and Axelsson et. al. (1965) reported that removal of dextrose abolished tension responses to electrical activity while the membrane was depolarized and increased its spike discharge for a considerable time. Glycogen stores (Bueding and Bülbring, 1964) however, under identical conditions were depleted very slowly and the process could be accelerated only by simultaneous anaerobiosis. They could, moreover, be virtually completely depleted (Bueding and Hawkins, 1964) under the substrate-free, anoxic conditions without completely abolishing contractile responses. These findings would suggest that the phosphorylase activity is under very weak feedback control from tissue energy levels in physiological conditions but that it can be activated in conditions of very severe energy depletion and that virtually all glycogen contents are labile. The alternative interpretation of these findings would be that considerable compartmentalization of energy stores exists, with the energy of carbohydrate metabolism directly supporting membrane polarization and contractile responses while phosphorylase is activated by decreases in a larger energy pool which has

a longer turnover time.

The trachealis muscle, in contrast, maintains normal contractility for many hours in an oxygenated dextrose-free environment. Under these conditions glycogenolysis is slightly increased (compared to its rate in a dextrose-containing solution) but high energy phosphates are not measureably decreased. In hypoxia, however, glycogenolysis was activated rapidly with the rate reflecting the depression of contractile function. This observation suggests that in the trachealis muscle glycogenolysis may be intimately controlled by tissue energy levels.

The effect of insulin on glycogen content was of special interest since in aerobic rabbit stomach and bovine mesenteric arteries significant increases in glycogen content have been reported (Lundholm and Mohme-Lundholm, 1963) with insulin equilibration. In the present studies, insulin did not prevent a net glycogenolysis of aerobic trachealis muscles. On the other hand, a marked glycogen-sparing effect of insulin was noted in hypoxic muscles. These results suggest that glucose entry into the muscle cell is not rapid enough to support the increased glycolysis in hypoxia, with the result that glycogenolysis is increased while in the presence of insulin the dextrose transport system can be facilitated enough to protect partially the glycogen stores of the muscle.

C. EFFECT OF HYPOXIA ON HIGH ENERGY PHOSPHATE STORES

The concentrations of high-energy phosphates in smooth muscle are considerably lower than those in skeletal and cardiac muscle (table 5) and appear, generally, to be related to the state of activity of the muscle in vivo.

TABLE 5

High Energy Phosphate Stores in Muscles

Tissue	CP (μmoles/g wet wt)	ATP (μmoles/g wet wt)	Reference
human skeletal muscle	13.3	6.2	Cretius, 1957 a,b
guinea pig taenia coli (with dextrose)	3.33	1.47	Bueding, et. al, 1967
(without dextrose)	0.92	0.79	Bueding, et. al, 1967
bovine mesenteric artery	0.66	0.91	Beviz, et. al., 1965
human uterus term	2.7	2.4	Cretius, 1957 a,b
non-pregnant	1.2	1.2	Cretius, 1957 a,b
canine trachealis (without dextrose)	0.94	1.12	(present study)

(Note: Unless otherwise stated, muscles were equilibrated with dextrose)

Thus the taenia coli which exhibits spontaneous activity continuously and pregnant (term) uteri have relatively high ATP and CP contents in comparison with non-pregnant uteri, vascular smooth muscle and trachealis whose activity is not as great.

The pattern of changes in high energy phosphates in hypoxia is similar to that found in hypoxia of cardiac muscle (Feinstein, 1962), with creatine phosphate being most markedly affected and ATP depressed to a lesser extent. It is also evident that adenosine nucleotides are converted to other compounds in hypoxia since the decrease in ATP is greater than the increases in ADP and AMP. This is probably due to conversion of AMP to hypoxanthine, and inosine monophosphate thus preventing inhibition by high AMP levels of the myokinase reaction to reform ATP (Imai et. al., 1964).

The observation that in trachealis muscle contractile function is much more closely correlated with creatine phosphate than with ATP content is consistent with the concept of compartmentalization of ATP stores (Opie, 1969a; Davies, 1965). Observations such as these have led to speculation that the ATP immediately available for contraction and in equilibrium with CP is a small compartment with a high turnover rate and is in equilibrium with a large, relatively inaccessible ATP compartment.

I. The Lohmann reaction in smooth muscle

While the pattern of depletion of high energy phosphates in hypoxia of the trachealis resembles that of skeletal and cardiac muscle where the Lohmann reaction is rapid, Daemers-Lambert (1964) and Lundholm and Mohme-Lundholm

(1964) have obtained evidence that in vascular smooth muscle (bovine carotid and mesenteric arteries, respectively) direct glycolytic and oxidative re-synthesis of ATP during contraction is primarily responsible for maintenance of ATP levels and that the total energy utilized is up to three times the total stores of pre-formed high energy phosphates. In the present study we have not, however, directly assessed the relative roles of ATP resynthesis via metabolism and the Lohmann reaction. However, the general correlation between CP content and contractile function (and lack of a similar correlation with ATP content) observed in the taenia coli (Born, 1956) and in this study on the trachealis would suggest that perhaps the mechanisms found in arterial muscle are not applicable to these muscles.

D. RIGOR IN SMOOTH MUSCLE

There has been some question as to the occurrence of rigor mortis in smooth muscle, and specifically on the similarity of the rise in resting tension observed in the present study to rigor. In most experiments involving metabolic poisoning of smooth muscle (Lundholm and Mohme-Lundholm, 1966; Born, 1956; Csapo and Gergely, 1950) active and resting tension have been observed to decrease and no increase in resting tension has been observed. A possible exception was the irreversibility of K^+-stimulated contraction of iodoacetate-poisoned bovine carotid arteries as the potassium was washed out (IAA maintained) observed by Daemers-Lambert (1964). He did not, however, report a rise in spontaneous tension with IAA and, thus, the phenomenon is difficult to interpret.

Although the ATP content of the trachealis is somewhat decreased at the time when the rise of resting tension is observed, it is still almost twice as high as the level found by Infante and Davies (1965) for skeletal muscle in rigor. The rapid reversibility of the phenomenon in trachealis on the addition of dextrose or oxygen would also argue against considering the rise in RP to be rigor. The data presented in this study also suggest that the observed rise in resting tension may be stimulated by rising intracellular free Ca^{++} levels (discussed below).

E. EFFECT OF HYPOXIA ON CALCIUM CONTENT AND EXCHANGE KINETICS

I. Calcium content

Since ion pumps in muscle normally maintain a very low ionized calcium level intracellularly against a considerable electrochemical gradient it was important to the study of the effects of hypoxia to monitor net calcium movements in hypoxia with and without the presence of extracellular Ca^{++}. Special care had to be taken in the measurement of tissue calcium content so that changes induced by experimental procedures were not lost in artefacts in the method. Thus phosphate-free Ringers solution containing a relatively low concentration of external Ca^{++} was used to prevent the accumulation of this ion in micro-precipitates of calcium phosphate complexes intracellularly (Goodford, 1967; van Breemen et. al.; 1966) and to prevent phosphate interference in atomic absorption spectrophotometry.

The normal absolute values of calcium contents of various smooth muscles are difficult to compare because of the wide variety of combinations and

concentrations of calcium and phosphate ions used in equilibration (Hurwitz et. al., 1969; van Breemen et. al., 1966) equilibration times, and procedures designed to deplete tissue calcium prior to experimental procedures (Hurwitz et. al., 1969) or before analysis (Hurwitz, Joiner and von Hagen, 1967). In spite of these differences, the normal resting value of 1.60 $\pm$ 0.12 mmoles/kg wet weight obtained in the trachealis is comparable to values obtained in the guinea pig taenia coli (1.7 $\pm$ 0.04 mmoles/kg wet weight, Lüllman and Mohns, 1969) diethylstilbestrol-treated rat uterus (2.25 mmole/kg wet weight, van Breemen, et. al., 1966) rabbit (1.57 $\pm$ 0.14 mmoles/kg wet weight, Briggs, 1962) and frog stomach (1.54 $\pm$ 0.13 mmoles/kg wet weight, Bozler, 1963)

The increase in tissue calcium content in hypoxia with normal calcium externally observed in this study was similar to the increase observed in rat uteri poisoned with IAA (van Breemen et. al., 1966) though in the present study the latency was shorter (< 60 min cf 70 min) and the absolute calcium uptake small due to the short (60 min) hypoxic exposure. A similar uptake of calcium by taenia coli in anaerobic, glucose-free medium has been reported by Bauer et. al. (1965). Since the object of these experiments was to assess the possible significance of calcium accumulation in activation of the contractile apparatus, it was important that the muscles be analyzed within a short time after the increase of resting tension in hypoxia.

Of interest was the observation of a decreased tissue calcium content of hypoxic muscles equilibrated in Ca^{++}-free Ringers solution as compared

to normoxic controls. Similar observations have not been reported for other smooth muscles. This observation, when considered together with the increased calcium of hypoxic muscles in normal calcium Ringer suggested to us that two processes, net Ca^{++} entry from the ECF and the release of Ca^{++} from bound intracellular sites were occurring simultaneously both of which could potentially raise the intracellular free-Ca^{++} concentration. These findings are consistent with the hypothesis that active binding or transport of calcium occurs at some intracellular site as well as at the sarcolemma. Since the cell membrane is permeable to calcium and the intracellular space represents a relatively large sink for this ion it is logical to suppose that the change in total calcium content after inhibition of both pumps should depend on the external calcium concentration as these results suggest.

Although the net uptake of calcium was sufficient to activate the contractile elements, this uptake does not necessarily imply an increase in free intracellular calcium concentration as van Breemen et. al. (1966) and Hurwitz et. al. (1967) have demonstrated a net uptake of calcium by uterus and ileal smooth muscle when incubated with a solution containing a high calcium concentration but a rise of resting tension was not observed. Since it was not feasible technically, to measure free-Ca^{++} levels intracellularly, we decided to measure Ca^{45} fluxes with a view to detecting some change in a kinetically defined calcium compartment.

II. Exchange kinetics

Net Ca^{45} efflux (figure 8) and desaturation curves have been analyzed by fitting a suitable number of exponentials (usually two or three) to the data obtained in experiments on a number of muscle preparations (Table 6). While

TABLE 6

Kinetically Defined Calcium Compartments in Smooth Muscle

Tissue	Rapid $t_{1/2}$ (min)	Medium $t_{1/2}$ (min)	Slow $t_{1/2}$ (min)	Reference
Frog sartorius	3	--	500	Shanes and Bianchi, 1959
Guinea pig atria	4.5	86 (168)	inexchangeable	Winegrad and Shanes, 1962
Guinea pig taenia coli	<3 min	3	30	Schatzman, 1961
Cat intestine	--	8	60	Sperelakis, 1962
Rat uterus	compartmental analysis impossible			van Breemen, et.al., 1966
Canine trachealis	0.7	12.2	471	(present study)

such an analysis is not always possible (van Breemen et.al., 1966) successful exponential analyses are usually interpreted as arising from homogeneous, well-mixed compartments with first order kinetics and visualized as representing some structural component of muscle. Thus the very rapid initial component is thought to arise from calcium in the extracellular space, the medium compartment from calcium loosely bound to the cell surface and connective tissue and the intracellular calcium exchanging more slowly still (Schatzmann, 1961). While such a visualization is attractive and useful to a certain extent, it is almost certainly an oversimplification since the muscle cells have a variety of calcium stores and Ca^{++} is a membrane stabilizer as well as activator of the contractile apparatus. Hence the possibility that some cellular calcium is in series with other calcium binding sites (Daniel, 1965) must be seriously considered. Series and series-parallel relationships of calcium fluxes would complicate considerably the evaluation of Ca^{45} efflux curves. The fact that Ca^{45} exchange is, to a certain extent dependent on external Ca^{++} (Bianchi and Shanes, 1960; Hudgins and Weiss, 1969) may be a result of these relationships.

The reason for the classification of slowly exchanging component of uterine calcium as "inexchangeable" (van Breemen, et.al., 1966) when a definite slope in their Ca^{45} uptake curve (fig. 1 in above reference) can be discerned, is not apparent. Similarly Bauer et.al. (1965) have not characterized the "slowly exchanging" calcium compartment of taenia coli, the size of which, interestingly was very temperature-dependent.

No significant difference in these compartments could be discerned in hypoxic muscles, however. This failure was undoubtedly due in part to the great degree of experimental scatter. Since we had previously demonstrated a loss of calcium from a cellular store in hypoxic muscles equilibrated in a Ca^{++}-free environment, this release should have been demonstrable kinetically as well. The time course of this Ca^{45} efflux was of additional interest as it related to the rise in resting tension. In subjecting the muscle to hypoxia at a time when the efflux rate had become low and quite constant a marked increase in Ca^{45} efflux was observed at the same time that the increase in resting tension occurred (Fig. 9). Van Breemen and Daniel (1966) used a similar technique to demonstrate the effect of potassium on Ca^{45} efflux from the rat uterus. Although we know from atomic absorption data on tissue calcium levels that under these conditions the tissue accumulates calcium, this observation confirms that calcium is being released from a compartment with a long half time which has, thus, retained a relatively high specific activity of Ca^{45}. If this Ca^{45} is being released from the most slowly exchanging compartment as appears likely, it should be noted that, because of the relatively short Ca^{45} "loading" period, this compartment is incompletely labelled and thus that the increased Ca^{45} efflux observed underestimates (by about 88%) on the basis of specific activity the actual amount of calcium released from this compartment. In any case, it is clear that calcium is being released from some bound compartment in hypoxia and its time course indicates that it could be related to the rise in resting tension.

The interpretation we have given our results emphasizes the probable involvement in the rise of resting tension of decreased energy stores for the active transport of calcium. Hinke (1965), however, has suggested that the calcium binding capacity of the bound calcium store in arterial muscle is pH-dependent with unbinding being facilitated either directly through increased solubility of Ca^{++}-complexes, or indirectly through inhibition of a Ca^{++} pump at low pH. Since anaerobic glycolysis produces lactic acid it could be argued that the H^{+} ion is responsible for the observed tension changes in the present studies. If this, in fact, were the case, it would predict that these effects should be abolished by inhibitors of glycolysis such as iodoacetate and potentiated by the addition of extracellular dextrose. These predictions could not be demonstrated, however, and it was observed that metabolic inhibition by itself could elicit the rise in tension and the addition of dextrose rapidly reversed the effect of hypoxia.

These observations contrast with those of Goodford (1965 a) on anoxic, glucose-free and DNP-poisoned taenia coli in which no increase of Ca^{45} efflux could be detected, although inadequate resolution may have been at fault. Although van Breemen et.al. (1966) observed calcium accumulation with IAA and DNP-poisoning of rat uterus, they also could not discern a decreased efflux with this treatment. This observation was the basis of their active exclusion hypothesis for the membrane calcium pump. The explanation of these differences may be that the taenia coli and uterus have relatively small

intracellularly bound calcium stores and/or that they exchange more rapidly. Independent evidence that this may be the case is the absolute calcium dependence of acetylcholine stimulated contraction in these muscles (Edman and Schild, 1962; Bozler, 1969; Bülbring and Kuriyama, 1963 b) compared to the relative insensitivity of the trachealis to this ion (fig. 10).

F. MODEL OF CALCIUM STORES

We then became interested in confirming by an independent method the active nature of this binding and the relationship of this bound store to external calcium. In investigating the responses of a normoxic muscle to acetylcholine in the absence of external calcium (fig. 10) we found that under these conditions it contracted phasically after which only a small amount of tension was maintained. If the calcium-free conditions were then maintained while the acetylcholine was washed out and the muscle given a short rest, it could, on restimulation produce another phasic contraction whose peak exceeded the steady state value of the previous contraction. Acetylcholine was ineffective after the rise of resting tension in hypoxia (Ca^{++}-free) however. If the normoxic muscle contractions reflected the intracellular free-Ca^{++} concentration, these observations would suggest that acetylcholine mobilized a limited amount of intracellular calcium, transiently raising the Ca^{++} concentration and activating the contractile apparatus, resulting in the initial peak of the phasic contraction. However, since many intracellular substances such as protein and ATP have an affinity for Ca^{++}, the pCa presumably increased as Ca^{++} was subsequently bound by these substances and the contraction decreased to a low level. It

would also appear that in the 10 minute rest period the internal calcium pump, through raising the intracellular pCa recovered much of the calcium that had become bound to tissue elements other than the tropomyosin system in the previous contraction (some of the calcium was probably lost through the cell membrane thus explaining the decrease in the peak of the second contraction as compared to the peak of the first contraction).

The other possible explanation for the phasic nature of the acetylcholine stimulated contraction in a Ca^{++}-free solution is that high energy stores are not adequately maintained to sustain a tonic contraction. This possibility, however, does not seem likely in view of the fact that this substance can stimulate a contracture which, in the presence of calcium is fully maintained for at least one hour. (Kroeger and Stephens, unpublished observations).

Since the bound store evidently recovered most of the Ca^{++} initially released, as judged by the relative heights of successive acetylcholine contractures in Ca^{++}-free solution, this observation suggested to us that the bound store was functionally an intracellular structure and did not have direct access to the extracellular space. If this were the case, it would seem strange that a structure which normally needed to maintain practically no electrochemical gradient with the ECF should be so resistant to depletion by low extracellular calcium concentrations. On the other hand, since acetylcholine and related compounds probably do not traverse the cell membrane (Durbin and Jenkinson, 1961) it seems likely that the sequestered calcium store must be rather superficially located.

The simplest explanation of our observations on the trachealis muscle would be that the sequestered calcium compartment is in series with the superficial, membrane-stabilizing calcium but that calcium entering in response to depolarization need not enter via the sequestered stores (i.e. the series-parallel model). Additional evidence we have obtained in this regard is that, while K^+-stimulated responses are more readily lost in Ca^{++}-free media, acetylcholine responses are regained more slowly after severe calcium depletion. These observations confirm for this muscle similar responses reported for vascular smooth muscle (Hinke, 1965) and rat uterus (Daniel, 1963). The concept of these stores being functionally in series as far as Ca^{45} efflux studies are concerned raises an objection to the method of compartmental analysis employed. Insofar as the Ca^{++} released by compartments with long half times must traverse other compartments with characteristic half times, a lag is introduced thus further slowing the apparent kinetics of these more firmly bound stores. Since the relationship of the compartment half times measured in this study are such that each successive store is more than ten times as rapid as the previous one, the error introduced becomes academic.

No conclusions can be drawn from the present studies on the question as to whether the membrane calcium pump consists of an active exclusion or extrusion mechanism. Although it has been difficult to demonstrate active extrusion (van Breemen et. al., 1966) as it is likely to consist of a very small part of the total flux of this ion (Bozler, 1963) it might be

stated on the other hand that in order for an exclusion process to function adequately it would have to render the membrane totally impermeable to Ca^{++}. Since this is certainly not the case in smooth muscle, the process of active extrusion of Ca^{++} remains to be demonstrated in this preparation.

In conclusion, it appears that in hypoxic trachealis muscle the loss of energy stores in glycogen and high energy phosphates are related to the loss of active tension and the development of a small additional resting tension. The latter has been shown to be closely related to changes in calcium metabolism in a manner consistent with the concept of inhibition of two sites of active transport for this ion such that in the presence or absence of external calcium the intracellular free-Ca^{++} concentration rises, activating the contractile apparatus.

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