SOME EFFECTS OF INCREASED EXTERNAL POTASSIUM ON FROG MUSCLE METABOLISM IN AEROBIC AND ANAEROBIC CONDITIONS

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To

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ABSTRACT

It has been known for about forty years that treatment of frog skeletal muscle with 10 to 20 mM KCl causes a maintained increase in metabolic rate without the development of contractile force. Hill and Howarth in 1957 reported that this increment in metabolism was almost completely abolished in O_2 - free conditions and concluded that the sustained treatment of the muscle with high K_o Ringer probably disrupted the normal metabolism. In the present study, effects of treatment of muscle with 15 mM K Ringer on certain aspects of muscle biochemistry were determined either in the presence or absence of O_2. Measurements of muscle glycogen, glucose and fructose-6-phosphate, lactate, creatine phosphate, and adenosine tri-, di- and mono-phosphate were performed and correlated with the effects of high extracellular potassium on anaerobic and aerobic muscle heat production. It is concluded from the experimental data obtained that the metabolic increment observed in aerobic and anaerobic conditions is apparently dissimilar in terms of ATP turnover and that treatment of aerobic muscle with 15 mM K Ringer probably impaired mitochondrial oxidative phosphorylation.

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I. INTRODUCTION

A. Review of Literature

In 1934, Hegnauer et al. reported that increased levels of extracellular potassium had the effect of increasing the O_2 consumption of frog skeletal muscle. The increase in QO_2 was observed to have a threshold of about 13 mM KCl, while the contracture, or mechanical threshold was 21 to 26 mM KCl (the threshold for stimulation of lactic acid production was 26 mM KCl). It was also reported that levels of extracellular potassium between 17 and 21 mM caused an increase in QO_2 which was maintained for over 5 hours.

In 1936, Solandt reported that an increased concentration of extracellular potassium caused the rate of heat production of frog skeletal muscle to increase. Smith and Solandt (1938) reported that:

1) the increase in rate of heat production was associated with a threshold of 8 to 12 mM KCl;

2) in the range 8 to 20 mM KCl, the maximum increase in the rate of heat production ($\dot{h}$) was proportional to the external potassium concentration (K_o) and that the increase $\dot{h}$ was maintained over a 5 hour period;

3) when K_o was increased to 20 to 120 mM there was a brief increase in $\dot{h}$ to levels greater than that due to 8 to 20 mM K_o, followed by a decrease to slightly less than the resting $\dot{h}$; and

4) the increase in $\dot{h}$ due to 8 to 20 mM KCl was not due to the development of muscle tension.

Summary: The earliest work indicated that increase in the extracellular concentration of potassium by an amount not sufficient to cause contraction could cause the metabolic rate of frog skeletal muscle to increase for long periods. The threshold for the stimulation of metabolism was at a K_o of about 10 mM, while the threshold for contraction was in the range of 20 to 25 mM K_o.

In 1957, Hill and Howarth reported findings that confirmed the results of Smith and Solandt. Hill and Howarth stated that the effect of increased K_o was to depolarize the muscle membrane and concluded that this depolarization somehow led to the increase in $\dot{h}$. They reported that depolarization of the muscle membrane to -65 mV (from a resting level of -90 mV) caused a slight rise in $\dot{h}$, while a maximum rise in $\dot{h}$ occurred at -50 mV (-65 mV and -50 mV correspond to 10 and 18 mM K_o respectively). Tension development did not occur till the muscle was depolarized to -45 mV (or K_o > 20 mM). The increase in $\dot{h}$ due to K_o = 18 mM was of long duration and was up to 40 times the resting rate. It was also found that an an anaerobic atmosphere (O_2 free) inhibited the increase in $\dot{h}$ (the increase in $\dot{h}$ due to K_o = 18 mM in anaerobic conditions was about 1/10 the increment seen in the presence of O_2).

A further observation of Solandt (1936) confirmed by Hill and Howarth was that calcium ions (Ca^{++}) antagonize the metabolic stimulation due to high K_o (10 to 20 mM). It was reported that Ca and K in equimolar concen-

trations caused little metabolic stimulation, while the addition of more K to such a solution caused the heat rate to rise. If K_o was increased to 10 to 20 mM, addition of Ca could cause the heat rate to fall to the basal level. It was concluded that the extracellular Ca concentration (Ca_o) could alter the threshold of the metabolic stimulation due to high K_o.

It is also reported that the rate of muscle heat production was increased by the addition of 15 to 25 mM rubidium chloride instead of increased potassium chloride. In terms of the effect on heat rate, 15 and 25 mM Rb^+ corresponded to 10 and 18 mM KCl respectively. It was noted that increased RbCl in the bathing solution had the effect of depolarizing the muscle membrane and that 15 and 25 mM RbCl caused the same degree of depolarization as 10 and 18 mM KCl.

Summary: Hill and Howarth's work indicated that the effect of increasing K_o was to depolarize the muscle membrane; thus, the threshold for the stimulation of metabolism was about -65 mV (10 mM K_o), and a maximal, sustained increase was seen at about -50 mV (18 mM K_o), while the threshold for contraction occurred at about -45 mV (20 to 25 mM K_o). An O_2- free atmosphere apparently inhibited the metabolic stimulation caused by 10 to 20 mM K_o. Ca ions were observed to have an antagonistic effect on heat rate since equimolar concentrations of Ca_o and K_o always caused the heat rate to come to the basal level. Substitution of Rb ions for K ions further indicated that the stimulus for metabolism due to high K_o was probably the

depolarization of the muscle membrane.

In 1959, Briner et al. reported findings concerning the effect of high K_o on the concentration of high energy phosphate compounds in sartorii of Bufo marinus. It was found that when K_o was equal to 20 mM, both the concentration of adenosine triphosphate and the ratio, creatine phosphate: creatine, significantly decreased. This change was not seen when K_o was equal to 10, 40 or 100 mM. It was also reported that 20 μM dinitrophenol had the same effect as 20 mM KCl. Briner et al. concluded from the results that the effect of 20 mM KCl might be the same as that of dinitrophenol, that is, interference with the process of oxidative phosphorylation.

Muller and Simon in 1960 reported results similar to those of previous authors; it was found that the rate of oxygen consumption (Q_{O_2}) of toad skeletal muscle increased with K_o greater than 10 mM and the maximum increase in Q_{O_2} occurred between 15 to 20 mM K_o. Muller and Simon concur with Briner et al. in concluding that the effect of 10 to 20 mM K_o is to interfere with mitochondrial function; more specifically, to uncouple the formation of high energy phosphate bonds from oxidative metabolism.

Summary: The studies of Briner et al., and Muller and Simon strongly suggest a defect in the process of ATP synthesis when muscle is depolarized by increasing K_o to a level of 20 mM K_o, that is, to the same level of K_o which was observed to cause a sustained increase in heat rate and Q_{O_2}.

The defect was thought to be mitochondrial, i.e., an interference with the process of oxidative phosphorylation (in a manner similar to that of dinitrophenol, a known uncoupler of oxidative phosphorylation).

In 1962, Novotny et al. investigated the effect of high K_o and caffeine on Q_{O_2}, and the modifying effect of certain drugs. It was reported that 18 mM K_o increased the Q_{O_2} by 8 to 10 times. Dinitrophenol (30 μM) had the same effect on Q_{O_2} as 18 mM K_o. The drugs physostigmine, phenobarbital, and procaine (in concentrations of 10^{-4} M, 10^{-4} M and 10^{-3} M respectively) completely abolished the increment in Q_{O_2} due to 18 mM K_o or 2 mM caffeine. Novotny et al. concluded that the increase in Q_{O_2} due to increased K_o or caffeine was caused by some change in the state of the muscle membrane.

In 1966, Novotny and Vyskocil reported findings concerning the effect of increased K_o on Q_{O_2} and calcium 'exchangeability' (the actual parameter measured was the intracellular concentration of labelled calcium after pre-exposure to 'hot' Ringer solutions with different levels of K_o; calcium exchangeability refers to the Ca^{45} content of the experimental muscle as compared to the control). It was found that curves relating Q_{O_2} and calcium exchangeability to K_o were qualitatively the same, but not quantitatively since 20 mM K_o caused a 7 to 8 fold increase in Q_{O_2} and a 2 to 3 fold increase in calcium exchangeability. Novotny and Vyskocil conclude that the effect of depolarization (increased K_o) and caffeine is to release bound

calcium into the sarcoplasm and increase the rate of utilization of high-energy phosphate bonds and, therefore, the rate of high-energy bond resynthesis (i.e. Q_{O_2}).

Summary: The work of Novotny and co-workers indicated that some modification of the muscle membrane was probably responsible for the increase in Q_{O_2} and heat rate caused by 10 to 20 mM K_o; drugs, described as local anaesthetics, known to have an action on the extracellular surface of cell membranes, completely abolished the increased Q_{O_2} due to high K_o. Other work established that calcium transmembrane fluxes were increased by depolarization, having a threshold of 10 mM K_o and a maximum, sustained increase at 18 mM K_o.

That depolarization and caffeine could alter transmembrane movements of labelled Ca and intracellular levels had been shown by researchers prior to 1966. Bianchi and Shanes in 1959 reported that stimulation of the muscle sufficient to cause contraction caused increased ^{45}Ca influx and efflux across the muscle membrane. It was also reported that when K_o was increased to 21.6 mM, the intracellular ^{45}Ca content doubled. Bianchi (1961) reported that 5 mM caffeine (a concentration sufficient to cause contraction) caused the intracellular ^{45}Ca content to triple. Feinstein (1963) reported that 5 mM caffeine increased both the rates of calcium influx and efflux (although influx was increased to a greater degree than efflux) and that these increases were abolished by local anaesthetics such as procaine.

In 1967, Van der Kloot reported data that re-emphasized the role of the membrane potential in alterations of the Q_{O_2}. It was found that increased Q_{O_2} was caused by increased levels of extracellular potassium, rubidium, and cesium, and that the increase in Q_{O_2} was related to the depolarizing ability of the various cations. The threshold for the stimulation of Q_{O_2} was again found to be about -65 mV while maximum stimulation occurred at about -50 mV. The increased Q_{O_2} due to 20 mM K_o was found to be maintained over a period of several hours (the stimulated Q_{O_2} was double the resting Q_{O_2} even after 16 hours) while increased Q_{O_2} due to 50 and 110 mM K_o were maintained for about 0.5 and 0.2 hours respectively (in agreement with Novotny et al., 1966). Van der Kloot concluded that the increased Q_{O_2} was probably due to increased levels of intracellular calcium which somehow caused increased adenosine triphosphate hydrolysis.

In 1969 Van der Kloot reported evidence which suggested that increased transmembrane Ca-flux per se was probably not the cause of the increased Q_{O_2} due to increased K_o. He reported that calcium ions could be replaced by nickel ions in the Ringer solution and that the response to increased K_o was not changed. The presence of either cation in millimolar quantities, however, was essential. Since nickel ions are not thought to have an intracellular action (i.e. cannot be sequestered by isolated sarcoplasmic reticulum and cannot interact with isolated actomyosin), and flux

rates are not altered by depolarization, Van der Kloot concluded that extracellular interaction of calcium (normally) and nickel ions (experimentally) with the muscle membrane is necessary for increases in Q_{O_2} to occur.

Summary: The work of Van der Kloot further established the role of the membrane potential as the cause of the increased Q_{O_2} and heat rate. The values for threshold (-65 mV) and maximal, sustained increase (-50 mV) are in agreement with Hill and Howarth (1957). It was suggested that the importance of extracellular calcium ions in mediating the increased metabolic rate was in their interaction with the muscle membrane.

B. Statement of the Problem

Theories regarding the mechanism of the increased Q_{O_2} and $\dot{h}$ due to increased K_o may be divided into two general categories; those which assume the effect to be physiological (explainable in terms of normal metabolic control), and those which assume the effect to be pathological (explainable by abnormal metabolic control). The former theories all assume that the increased metabolic rate is due to increased hydrolysis of ATP, while the latter theories assume that there is disruption of the process of mitochondrial oxidative phosphorylation. The theories assuming normal metabolic control will be considered first.

Horowicz and Gerber in 1965 measured one-way labelled sodium fluxes in single muscle fibers in the presence of increased K_o (and strophanthidin). They observed that 10^{-5} M strophanthidin inhibited about 80% of the sodium efflux but had no effect on sodium influx. It was postulated that most sodium efflux is due to an energy consuming 'pump'. While 2.5 to 7.5 mM K_o had no effect on flux rates, K_o between 7.5 and 15 mM increased the rate of efflux; with 15 mM K_o the efflux rate increased 2.5- to 3- fold over control. Strophanthidin (10^{-5} M) completely inhibited the potassium-stimulated efflux. The authors noted that the increment in Q_{O_2} due to 15 mM K_o reported by Hegnauer et al. (1934) was of the same magnitude as the increment in sodium efflux and that the stimulated Q_{O_2} also had a threshold of about 7.5 mM K_o. It was concluded that the

increased metabolic rate induced by high K_o is caused by depolarization of the muscle membrane, and that stimulation of the sodium pump by depolarization might be the specific mechanism leading to increased ATP hydrolysis, a suggestion made earlier by Keynes and Maisel (1954).

Hill and Howarth (1957) state that stimulation of the sodium pump is not a likely mechanism to explain the extra metabolism. They point out that if the large (10 to 40 times the resting $\dot{h}$), and long maintained (several hours), increase in heat rate were due to sodium extrusion, than the process must either be very inefficient or the amount of sodium extruded "enormous" (it is suggested that the muscle would be sodium-depleted by such an efflux rate in minutes, not hours). Yamada (1970) added 10^{-4} M strophanthidin to a muscle in solution with K_o of 10 mM and found that the $\dot{h}$ was unchanged (i.e. remained at a high level). Muller and Simon (1960) reported that there was no significant change in muscle sodium content after 4 hours in a 15 mM K_o solution. It is apparent that the increased metabolism cannot be due to depolarization-induced changes in sodium pump activity.

One of the earliest theories concerning the mechanism was that of Hegnauer et al. (1934) who postulated that K_o between 13 and 21 mM caused a slight contracture of the muscle (causing increased activity of the actomyosin ATPase, which would lead to increased metabolic rate).

The contracture theory has been refuted by several authors who all

claim to be unable to detect any mechanical change in the muscle during depolarization. Solandt (1936) and Kaye and Mommaerts (1960) stated that increased K_o sufficient to cause increased Q_{O_2} (10 to 20 mM K_o) did not produce any change in muscle length or resting tension. Smith and Solandt (1938) stated that while there was some twitching of the muscle immediately after the introduction of the high K_o solution, this ended in one or two minutes, and that no mechanical change could account for the long duration increment of heat production. Hill and Howarth (1957), using a very sensitive force transducer, were unable to detect any changes in tension except for some initial twitching of the muscle (which ended after 2 minutes in high K_o solution). They also concluded that no mechanical change was associated with the long duration increment in heat rate. On the other hand, a recent report by Marco and Nastuk, 1968, states that under conditions of 0.25 to 2.0 mM caffeine, or depolarization to about -65 mV, highly localized contractions of a few sarcomeres can be observed. These so-called 'sarcomeric oscillations' might cause increased heat rate and Q_{O_2}, and would perhaps be undetectable by force-measuring techniques.

Several authors in the last decade have hypothesized that the increased metabolic rate is due to increased ATP hydrolyzing activity caused by increased levels of free intracellular calcium ion. This theory is largely due to experiments of Bianchi and Shanes (1959) who showed that depolarization due to increased K_o resulted in increased calcium influx. Kaye and

Mommaerts (1960) proposed that the increased glycolytic rate seen in increased K_o was due to increased intracellular calcium acting upon and increasing the glycogenolytic activity of the phosphorylase system, which would have the effect of increasing substrate levels for oxidative metabolism (Drummond, 1967). Novotny and Vyskocil (1966) suggested that depolarization-induced increased levels of free intracellular calcium stimulated the calcium sequestering 'pump' of the sarcoplasmic reticulum (i.e. the muscle was attempting to decrease the level of free calcium). Increased pump activity, by definition, would cause increased hydrolysis of ATP. Van der Kloot (1967) simply postulated that increased levels of intracellular calcium (caused by depolarization) stimulated the activity of an unspecified ATP hydrolyzing system. Vos and Frank (1972), using a different approach, reach the same conclusion as Novotny and Vyskocil (1966), that is, that the increased metabolism due to depolarization is caused by increased activity of the calcium sequestering pumps.

All calcium theories assuming increased intracellular free Ca^{++} due to increased Ca^{++} influx seem to be refuted by Van der Kloot (1969). He reported that while the presence of extracellular calcium in millimolar quantities was normally essential for the maintenance of the increased metabolism, all extracellular calcium could be replaced with nickel ions (in the same concentrations) and the depolarization-induced increased Q_{O_2} was still present to the same degree. Nickel ions were effective in

replacing calcium even though:

1) nickel ions do not activate actomyosin ATP hydrolysis,

2) nickel ions are not sequestered by isolated sarcoplasmic reticulum fragments, and,

3) nickel ion transmembrane fluxes are not altered by depolarization.

Van der Kloot concluded that the importance of extracellular calcium was in its interactions with the muscle membrane (which was somehow essential for the increased metabolism).

The findings of Van der Kloot do not exclude Ca pump activity since it is possible that the source of the Ca for pump activation is internal. It is presently believed that internal Ca stores are the source for Ca required in the contractile event, and Vos and Frank (1972) have hypothesized that the increased metabolism may be due to processes occurring in normal relaxation.

A refutation of all theories assuming normal metabolic control is probably given by Hill and Howarth (1957). It was observed by these authors that the increment in heat rate due to high K_o in an anaerobic (O_2 free) environment was only about 10% of the increment seen in the normal, oxygenated muscle. That is, depolarization of the muscle with 18 mM K_o, for example, increased the heat rate in aerobic conditions some 20-fold over the 'resting' heat rate, while the same K_o in anaerobic

conditions merely caused the heat rate to double (It was also observed that the 'resting' heat rate of aerobic muscle was approximately double that of anaerobic muscle.). Since metabolic rate of skeletal muscle is normally (physiologically) regulated by levels of ADP and ATP, it is to be expected that in order to maintain the ATP pool at normal levels the ATP synthesizing reactions must run a certain rate for a given set of conditions. That is, if there was a stimulus such that the rate of ATP use were to increase by, say, 10 times, and given that the level of high energy phosphate (CP plus ATP) did not change very much during the period of the increased ATP use, then it must follow that the rate of ATP synthesis had increased by about 10 times during the period of stimulation. Thus, if normal metabolic control were to exist during conditions of depolarization with 10 to 20 mM K_o, the increment in metabolic rate observed in aerobic conditions would have to be observed in anaerobic conditions as well (in order to have equal rates of ATP synthesis). Proponents of ATPase theories and normal metabolic control are therefore forced to conclude that the rate of ATP use and synthesis during depolarization with 10 to 20 mM K_o is many times greater in aerobic as compared to anaerobic conditions.

A few authors have concluded that the depolarization-induced stimulation of metabolism is pathological in the sense that the normal metabolic control is disrupted. Briner et al. (1959) reported that high K_o (20 mM) significantly reduced the muscle ATP content and reduced the creatine

phosphate : creatine ratio from 1.7 to 0.3 over a four hour period. Dinitrophenol (20 μM) had the same effect as 20 mM K_o. No conclusions were drawn in this report. Muller and Simon (1960), continuing from the Briner (1959) report, stated that the stimulation seen in 10 to 20 mM K_o, "must be considered as an uncoupling of oxidation from energy consuming processes". They decided that the increased QO_2 was not due to increased work performed by the muscle but was indicative of a pathological state. Hill and Howarth (1957) also conclude that the stimulation of metabolism due to high K_o was not physiological and was perhaps a sign of cellular damage.

It is apparent from the literature reviewed here that the mechanism of high (10 to 20 mM) K_o- induced increased metabolic rate is not established. Indeed it is uncertain as to whether the cellular events occurring are those of a normal or of a damaged muscle. Arguments presented by Hill and Howarth (1957), and Muller and Simon (1960) suggest the latter situation to be true (assuming that disruption of oxidative phosphorylation represents a pathological situation, which is not necessarily true since it has been demonstrated that energy released by mitochondrial oxidation may be utilized for purposes of ion translocation in preference to phosphorylation; see Lehninger, 1970a. At this moment however, it is difficult to imagine that the mitochondria of partially depolarized muscle do spend hours in translocating ions when evidence has shown that only minor changes occur in ionic content of the muscle.) It has also been established that the

phosphorylating ability of mitochondria is more sensitive to damage than the ability to oxidize substrate (see, for example, Laudahn and Rasch, 1958) so there is some reason for hypothesizing that disruption of oxidative phosphorylation occurs during sustained partial depolarization of frog skeletal muscle.

The study reported in this thesis was undertaken to more thoroughly research the observation of Hill and Howarth (1957) in regard to the differential effect of high K_o in aerobic and anaerobic conditions, it being the present author's opinion that this observation constitutes the best reason for believing that depolarization by 10 to 20 mM K_o causes a situation of abnormal metabolic control. Measurements of rate of muscle heat production and levels of the high energy phosphates and certain substrates of carbohydrate metabolism were made in O_2- saturated and O_2-free conditions in solutions with normal and high potassium concentration. Measurement of oxidizable substrates was done to provide an indication of the metabolic rate at the level of cellular biochemistry since measurement of heat production is a highly-nonspecific index of metabolic rate. Measurement of the high energy phosphates was done to provide some idea as to the rates of ATP synthesis and utilization. If normal metabolic control were to exist in partially depolarized muscle then the observation of Hill and Howarth suggests that the rate of ATP synthesis (and use) in aerobic conditions would be 10 times the rate in anaerobiosis, which should be reflected in the measurements of the oxidizable substrates and high energy phosphates.

It is perhaps impossible to conclude if abnormal metabolic control is present in the experiments undertaken. Given that isolated frog muscle is 100% carbohydrate using, and that complete oxidation of one molecule of glucose from glycogen can result in the synthesis of 38 molecules of ATP (on the average only about 33 ATP are produced since perhaps 15% of glucose molecules are normally oxidized and reduced to lactic acid) and that 3 molecules of ATP can be synthesized through glycolysis alone, it follows that the rate of glycolysis must increase by 33/3 or 11 times in order to synthesize the same amount of ATP per unit time. It is obvious that if normal metabolic control exists in both aerobic and anaerobic conditions, and that there exists the same rate of ATP synthesis and use in these conditions, than the quantity of carbohydrate consumed by the anaerobic muscle must be much greater (11 times) than the amount consumed by the aerobic muscle. Therefore, if it was found that the amount of carbohydrate consumed in a given period of time was the same in both aerobic and anaerobic conditions, one would be forced to conclude that either,

1) the rate of ATP synthesis and use in the aerobic muscle was 11 times greater than in the anaerobic muscle (which assumes normal efficiency of ATP synthesis, i.e. mitochondrial intactness, or as the biochemists say, tightly coupled mitochondria), or,

2) the rate of ATP synthesis and use is the same, or similar, in both aerobic and anaerobic conditions and that the process of oxidative phosphory-

lation has been uncoupled. Unfortunately either conclusion 1) or 2) could be used to explain the differential stimulation of heat rate observed by Hill and Howarth since:

1) different rates of ATP synthesis would be reflected by different heat rates, and

2) a given rate of carbohydrate oxidation would give different heat rates in O_2 and N_2 due to the different degree of glucose catabolism by glycolysis and mitochondrial oxidation.

A comparison of the results of the data will be made in the Discussion and arguments presented that will attempt to show that conclusions can be made concerning the state of metabolism during partial depolarization.

II. METHODS

A. Introduction

Experiments were of 2 general types; measurement of heat production (in order to arrive at some estimate of the rate of metabolism of the muscle), and measurement of the concentration of various muscle metabolites (in order to obtain more specific information as to the effects on muscle metabolism). Thus, the study was essentially a correlation of muscle biophysics (heat production) and biochemistry (estimation of metabolites).

B. General Remarks

All experiments, unless otherwise indicated, were performed on sartorii from frogs of the species Rana pipiens. These frogs were obtained either locally or from a supply house in Wisconsin. Frogs obtained locally were generally much healthier and survived for several weeks if placed in a pool of deionized, distilled water (with about 1% normal Ringer solution added) in the refrigerator. Only healthy appearing frogs were used in any of the experiments.

The bathing solution used in all experiments was 'frog Ringer solution' containing either 2.5 or 15 mM KCl. Normal Ringer solution (designated 2.5 K Ringer) contained 2.5 mM KCl, 115 mM NaCl and 2 mM $CaCl_2$. The high K Ringer solution (designated 15 K Ringer) contained 15 mM KCl, 102.5 mM NaCl, and 2 mM $CaCl_2$. The solutions were buffered to pH 7.2 to 7.4 with NaH_2PO_4 - Na_2HPO_4 buffer (4.2 mM).

Temperature of the bathing solutions varied between 18 to 23°, a few degrees cooler than room temperature due to evaporation.

C. Measurement of Muscle Heat Production

Rate of heat production ($\dot{h}$) was determined using paired sartorii and a conventional thermopile, as described by Woledge (1961) and Clinch (1968).

The muscles are attached to the thermopile in such a way as to cover the 'hot' junctions. The 'cold' junctions, being insulated and thermostable, do not sense the temperature changes of the muscle. Changes in muscle activity cause changes in the muscle rate of heat production, thereby causing the muscle to become warmer or cooler. Change of temperature of the muscle, transmitted to the underlying 'hot' junctions of the thermopile alters the magnitude of the thermo-EMF existing between the 'hot' and 'cold' junctions. The output from the thermopile was led to a Kipp Micrograph recorder (maximum sensitivity = 2.5 μV/cm). The steady-state rate of heat production could then be calculated from the voltage read off the recorder (which is directly proportional to the temperature difference between the 'hot' and 'cold' junctions of the thermopile). The rate of heat production, $\dot{h}$, is:

$$\dot{h} = \frac{60 \cdot y \cdot c \cdot k}{\mu} \quad (\text{mcal} \cdot g^{-1} \cdot \text{min}^{-1})$$

where y = steady-state output of thermopile (μV)

c = specific heat of muscle

(0.88 cal $\times g^{-1} \times deg^{-1}$, Hill (1931))

k = cooling constant (sec^{-1})

μ = temperature sensitivity of thermopile

($\mu V \times mdeg^{-1}$)

k was determined separately in each experiment by briefly heating the muscle (with a short pulse at 50 k Hz sinusoidal current) and recording the time course of the subsequent temperature fall. It is important to establish that k is a constant for the muscles, that is, that the muscles do lose heat at a rate proportional to the temperature difference between the 'hot' and 'cold' junctions, since it is assumed in the $\dot{h}$ formula that the rate of heat gain is equal to the rate of heat loss (i.e. constant muscle temperature).

The paired sartorii were dissected and left attached to a portion of the pelvic bone. The muscles were then placed in unbuffered Ringer and refrigerated (5° C) for 1 to 2 hours prior to being placed on the thermopile. The thermopile and muscle were then placed in a special glass vessel which allowed the muscle and thermopile to be either bathed in Ringer solution or surrounded by air (Clinch, 1968). Since the bathing solution acts as a short circuit for the muscle heat, heat can only be measured with the muscle in air: the small 'heat' signal measured with the muscle surrounded by bathing

solution is referred to as an error signal and is generally a measure of the quality of the precautions taken to avoid the presence of spurious temperature gradients in the thermopile. Using our system the 'error in solution' signal was generally negligible. The glass vessel containing thermopile and muscles was immersed into a large Dewar flask which has been previously filled with water and allowed to come to thermal equilibrium. H_2O-saturated air was bubbled through the Dewar flask to provide mixing. Ringer-equilibrated O_2 or N_2 was bubbled through the thermopile vessel in order to create an aerobic or anaerobic atmosphere. The muscles generally required a 2 hour period to come to a constant rate of heat production (the 'resting' $\dot{h}$).

D. General Remarks Related to Biochemical Experiments

All biochemical experiments were done using separated, paired muscles; sartorii from a given frog were dissected and freed with both muscles attached to a common fragment of pelvic bone. The bone fragment could then be split with a razor blade to yield the individual muscles. A single frog, therefore, provided both control and experimental tissue. Since the mass of a single sartorius was 50 to 80 mg it was necessary to combine the muscles from four frogs to give sufficient tissue to perform the biochemical determinations.

Muscles used for glycogen and lactic acid determinations were placed

in unbuffered Ringer solution for one hour after dissection. Muscles used for creatine phosphate, adenosine tri-, di- and monophosphate, intramuscular lactic acid, glucose-6-phosphate and fructose-6-phosphate were dissected and left in the refrigerator in unbuffered Ringer overnight prior to experimental use. This latter precaution was absolutely necessary in order to establish resting levels of glucose and fructose-6-phosphate since levels of these metabolites vary over a hundred-fold between the metabolically resting and active state (Ozand and Nahara, 1964).

The experimental apparatus for the biochemical experiments consisted of four glass vessels interconnected by plastic tubing with one muscle set up in each vessel. Either pure O_2 or N_2 was then bubbled through the vessels (after first being bubbled through a vessel containing unbuffered Ringer in order that the gas be H_2O-saturated before passing into the experimental vessels).

Muscles were left in the experimental solutions for periods of up to 120 minutes. At the end of the experimental period muscles used for analysis of CP, ATP, ADP, AMP, lactate, G-6-P and F-6-P were removed from the baths and immediately frozen by squeezing the muscles with tongs precooled in liquid N_2. The frozen muscles were then transferred to a deep freeze (-85°C) and left for a few hours prior to deproteinisation and subsequent analysis (muscles which appeared to have thawn slightly were discarded, this being detectable by color change and loss of brittleness).

All enzymes were obtained from Sigma Chemical Ltd.

E. Estimation of Glycogen

The method for the estimation of muscle glycogen was obtained from Kroeger (1970), and is a modification of the technique of Montgomery (1957). A detailed analysis of the technique is given by Johnson and Fusaro (1970).

Glycogen content was determined using separated, paired muscles after they had been in Ringer solution for a period of 90 minutes. At the end of the experimental period the muscles were quickly blotted, weighed and then dropped into 2 ml of hot potassium hydroxide in small (3 ml) test tubes. The tubes were then placed in a boiling water bath for about 45 minutes for complete tissue digestion. After the tubes had cooled to room temperature, 0.050 ml of saturated sodium sulfate solution and 2.0 ml of absolute ethanol were added. The tubes were then returned to the hot (about 80° C) water bath for a few minutes till the glycogen had coagulated. Tubes were then centrifuged at slow speed (4,000 to 5,000 rpm) for 15 minutes and the supernatant was discarded. The precipitate was then solubilized in 2.0 ml of water, after which 0.030 ml of phenol reagent and 3.0 ml of sulfuric acid were added. After the tubes had cooled to room temperature, the absorption was measured at 490 mμ as compared to a water blank. The absolute amount of glycogen was then determined from a

previously prepared standard curve.

Reagents:

1. 30% (weight/volume) aqueous potassium hydroxide solution
2. reagent-grade sulfuric acid (> 95% purity)
3. phenol solution (80 g phenol plus 20 ml DDW)
4. absolute ethanol
5. glycogen stock solution (1 mg glycogen/1 ml DDW)

The phenol used must be of a 'pure' grade; crystals must be pure white. If the crystals are pink then the phenol should be redistilled. It is also important that the glycogen used for the stock solution be as 'pure' as possible in order to obtain a replicable standard curve.

F. Preparation of Deproteinized Muscle Extract for the Determination of Creatine Phosphate, Adenosine tri-, di- and monophosphate, Lactate, Glucose-6-phosphate and Fructose-6-phosphate

Muscles were removed from the deep freeze, weighed, and placed in mortars precooled with a small volume of liquid N_2. The frozen muscles were then powdered by grinding with pestle and mortar. After powdering, 10 to 30 ml/g muscle of 6% perchloric acid were added, depending on the weight of the muscles; usually yielding a volume of 6 to 8 ml. Since the perchloric acid usually froze in the mortar, it was necessary to melt the mixture by stirring with a glass stirring rod. Next the contents of the mortar were transferred to centrifuge tubes (precooled in ice) and centrifuged at

5,000 rpm for 10 minutes. The supernatant was then transferred to a beaker in ice and, with continuous stirring, sufficient 3 M potassium carbonate was added to bring the pH to about 7.5 (as measured with pHydrion paper). This mixture was transferred to ice-cooled centrifuge tubes and spun at 5,000 rpm for 10 minutes. The supernatant so obtained was then analyzed for the various metabolites. This technique was obtained from Fedelesova (private communication) and is a modification of the technique of deproteinization described by Bergmeyer (1963).

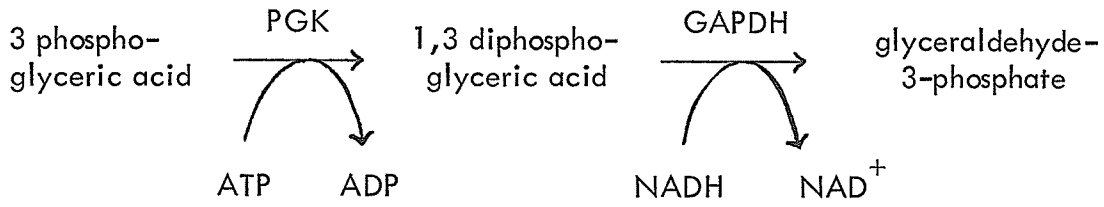
Reagents:

1. potassium carbonate (3 M K_2CO_3)
2. perchloric acid (6% w/v $HClO_4$)

G. Spectrophotometric Estimation of Creatine Phosphate, Adenosine tri-, di- and monophosphate

(a) The method for the estimation of creatine phosphate (CP), adenosine tri-, di-, and monophosphate (ATP, ADP and AMP) was that of Fedelesova (private communication) and was a modification of techniques outlined by Bergmeyer (1963).

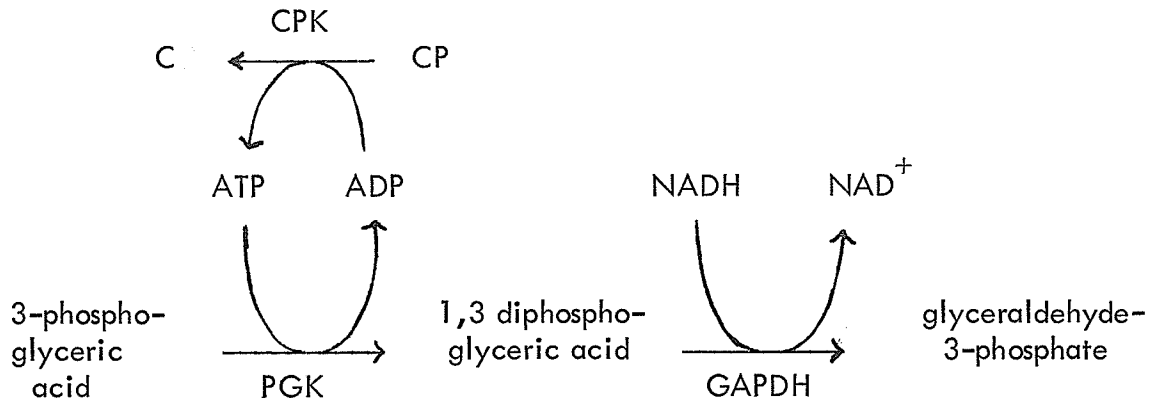
The estimation of ATP was based on the reactions catalyzed by phosphoglycerokinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH):



The concentration of ATP in the muscle extract was determined by adding 0.020 ml of glyceraldehyde phosphate dehydrogenase and 0.020 ml of phosphoglycerokinase to a cuvette containing 2.0 ml of buffer, 0.5 ml of muscle extract, 0.050 ml of reduced nicotinanide adenine dinucleotide, 0.100 ml of 3-phosphoglyceric acid solution and 0.020 ml of cysteine solution. The reaction (i.e. conversion of NADH to NAD) was followed in the spectrophotometer to completion at 340 mμ (the absorption maximum for NADH) and the concentration of ATP in the muscle was calculated from the change in absorption (ΔE), the ratio of volume of muscle extract to total volume of cuvette contents, and the original dilution of perchloric acid per gram of muscle.

$$\text{ATP}_{\text{in muscle}} = \Delta E/\varepsilon \times \frac{\text{muscle extract volume}}{\text{total cuvette volume}} \times \text{HClO}_4 \text{ dilution}$$

(b) The estimation of CP was based on the reactions catalyzed by creatine phosphokinase (CPK), phosphoglycerokinase (PKG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH):



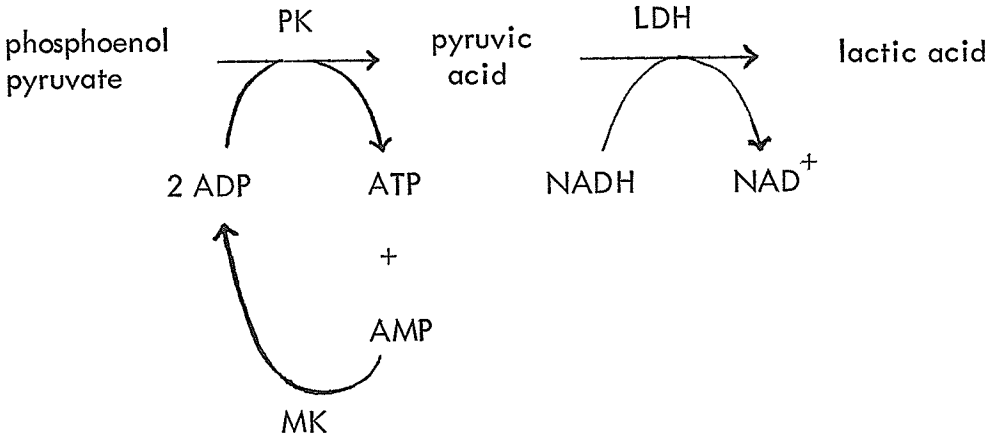
The concentration of CP in the muscle extract was determined by adding 0.010 ml of creatine phosphokinase to a cuvette containing exactly the same components used in the ATP determination, except that 0.1 ml of muscle extract was used. From the change in absorption at 340 mμ the concentration of CP was calculated (as for ATP).

Reagents:

1. buffer, pH 7.6 (100 mM triethanolaminic hydrochloride, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA, 0.3 mM ADP, adjusted to pH 7.6 with 2 N NaOH)
2. 3-phosphoglyceric acid (150 mM 3-PGA)
3. reduced nicotinamide adenine dinucleotide (3 mM NADH)
4. cysteine (150 mM cysteine), or dithiolerythritol (150 mM DTE)
5. glyceraldehyde phosphate dehydrogenase (4 mg/ml GAP-DH)
6. phosphoglycerol kinase (1 mg/ml PGK)
7. creatine phosphokinase (20 mg/ml CPK)

(c) The estimation of ADP and AMP was based on the reactions

catalyzed by lactic dehydrogenase (LDH), pyruvate kinase (PK) and myokinase (MK):



The concentration of ADP in the muscle extract was determined by adding 0.020 ml of lactic dehydrogenase and 0.020 ml of pyruvic kinase solution to a cuvette containing 1.0 ml of buffer, 1.0 ml of muscle extract, 0.050 ml of NADH solution and 0.150 ml of phosphoenol pyruvate solution. The reaction was followed to completion in the spectrophotometer at 340 mμ and the concentration of ADP was calculated from the change in absorption.

The concentration of AMP was determined by adding 0.020 ml of myokinase solution to the ADP cuvette after the reaction with lactic dehydrogenase and pyruvate kinase had gone to completion.

Reagents:

1. buffer, pH 7.5 (100 mM triethanolamine hydrochloride, 0.4

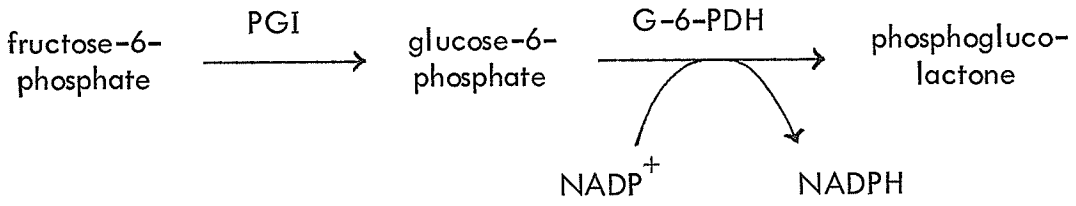
mM $MgSO_4 \cdot 7H_2O$, 0.1 mM EDTA, 3 mM K_2CO_3, adjusted to pH 7.5 with 2 N NaOH)

2. phosphoenol pyruvate (150 mM PEP)
3. reduced nicotinamide adenine dinucleotide (3 mM NADH)
4. lactic dehydrogenase (1 mg/ml LDH)
5. pyruvate kinase (1 mg/ml PK)
6. myokinase (5 mg/ml MK)

H. Estimation of Glucose-6-phosphate and Fructose-6-phosphate

The method used for the estimation of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) is a modification of the technique of Hohorst (1963).

The estimation of glucose-6-phosphate and fructose-6-phosphate was based on the reactions catalyzed by phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G-6-PDH):



The concentration of G-6-P in the muscle extract was determined by adding 0.020 ml of glucose-6-phosphate dehydrogenase solution to a curvette containing 1.0 ml buffer, 1.0 ml muscle extract, 0.020 ml

NADP solution and 0.020 ml magnesium chloride solution. The reaction was followed to completion in the spectrophotometer at 340 mμ. The concentration of G-6-P in the muscle in terms of μmoles/g could then be calculated from the change in absorption, the sample dilution, and the weight of the muscle as for ATP.

The concentration of F-6-P in the muscle extract was determined by adding 0.020 ml of phosphoglucose isomerase solution to the cuvette described above after the reaction with glucose-6-phosphate dehydrogenase had gone to completion.

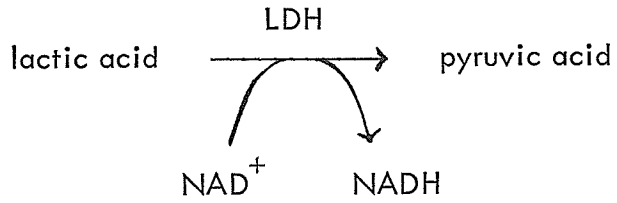
Reagents:

1. triethanolamine buffer, pH 7.6 (0.4 M triethanolamine hydrochloride, adjusted to pH 7.6 with 2 N sodium hydroxide)
2. magnesium chloride (0.5 M $MgCl_2$)
3. nicotinamide adenine dinucleotide phosphate (0.02 M NADP)
4. glucose-6-phosphate dehydrogenase (1 mg protein/ml)
5. phosphoglucose isomerase (0.5 mg protein/ml)

I. Estimation of Lactic Acid

The method used for the estimation is a modification of that of Hohorst (1963).

The estimation of lactic acid was based on the reaction catalyzed by lactic dehydrogenase:



(a) The concentration of lactic acid in the deproteinized muscle extract was determined by adding 0.020 ml of LDH to a cuvette containing 1.000 ml of buffer, 1.000 ml of muscle extract, and 0.020 ml of NAD solution. The reaction was followed to completion at 340 mμ (requiring 10 to 20 minutes) and the concentration of lactate calculated from the change in absorption (as for ATP).

(b) Lactic acid in the bathing solution was determined using split-paired muscles while the muscles were in solution. Muscles were set up in vessels of 25 ml volume. At 30 minute intervals, 2 ml of the bathing solution were removed (by syringe). Volume was kept constant by replacing with 2 ml of stock solution. At the end of the experimental period the muscles were blotted, weighed and discarded.

The concentration of lactic acid was then determined by adding 0.020 ml of LDH to a cuvette containing 0.90 ml of buffer, 0.100 ml of NAD, 0.50 ml of sample bathing solution and 0.50 ml of deionized, distilled water. Total volume of the cuvette was 2.02 ml. The reaction was then followed on the spectrophotometer at 340 mμ. From the change in absorption ($\triangle$E), the dilution of the sample, the weight of the muscle, and the time

at which the sample was taken, the rate of lactic acid production in terms of μmoles/gram wet muscle/hour was determined.

Reagents:

1. hydrazine-glycine buffer (0.4 M hydrazine, 1 M glycine, adjust pH to 9.5 with 2 M NaOH)
2. nicotinamide adenine dinucleotide (about 0.05 M)
3. lactic dehydrogenase, LDH (about 5 mg protein/ml)

III. RESULTS

A. Heat Production

The results of a typical experiment in which heat production is measured is shown in Figure 1. The resting rate of heat production was about 2 mcal $\cdot g^{-1} \cdot min^{-1}$, a value similar to resting heat rate usually found for frog sartorius muscle (Hill and Howarth, 1957; Yamada, 1970). Introduction of the 15 mM K_o Ringer caused an apparent immediate increase in heat rate with a new steady state heat rate being seen within about 45 minutes. The heat rate in 15 mM K Ringer was about 13 mcal $\cdot g^{-1} \cdot min^{-1}$, or 6 to 7 times the resting heat rate. Return of normal (2.5 mM K_o) Ringer caused the heat rate to return to resting levels apparently within 15 to 20 minutes. Introduction of pure N_2 gas into the muscle bath (i.e. anaerobiosis) caused the resting heat rate to decrease to about 50% of the aerobic heat rate. Treatment of the anaerobic muscle with 15 mM K Ringer caused the heat rate to increase about two-fold (in comparison to the 6 to 7 fold increment seen in aerobic conditions). The decreased increment in heat rate due to high K_o seen in anaerobiosis is not due to muscle fatigue since the experimental procedure can be reversed (anaerobic depolarization proceeding aerobic) and similar results obtained. Treatment of Rana pipiens sartorius with 15 mM K Ringer aerobically in other experiments on the average caused the heat rate to increase 6 fold, (with a 4 to 10 fold increment being the range of values); similar treatment of the anaerobic muscle caused no more thana doubling of heat rate. In two experiments in which sartorii

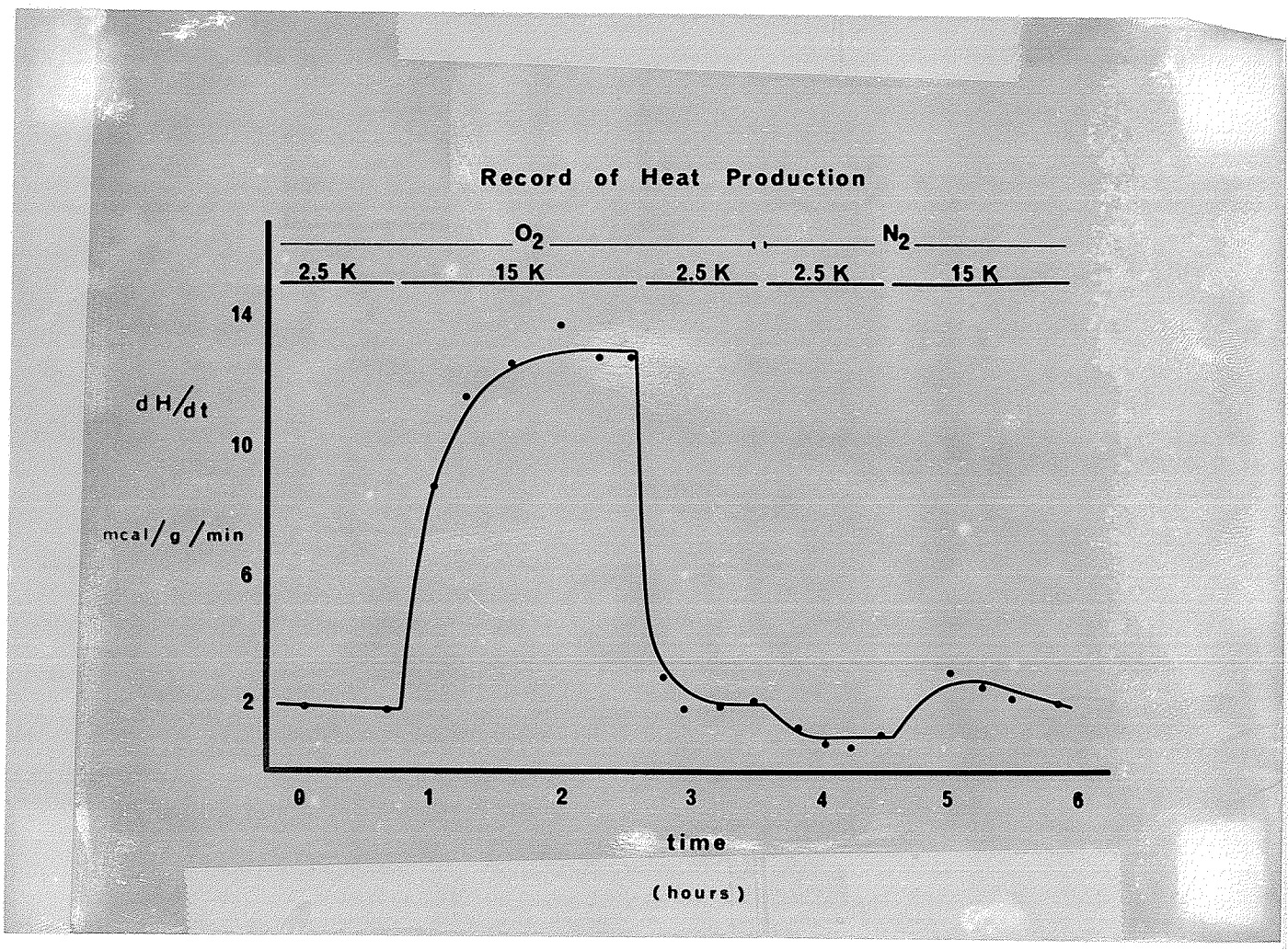


FIGURE 1. The rate of heat production in terms of mcal $\cdot g^{-1} \cdot min^{-1}$ is plotted versus time in hours. The horizontal bars at the top refer to the length of time the muscle was exposed to each of the experimental conditions, thus the muscle was exposed to 15 K Ringer and O_2 for about 2 hours for example.

of Rana temporaria were used, the aerobic increment in heat rate due to 15 mM K_o was 20 and 24 fold.

B. Glycogen Content

Results of the experiments in which muscle glycogen was measured are summarized in Table I and presented graphically in Figure 2. Variability in glycogen content in different frogs is obviously high (from 8 to 16 mg/g wet muscle in conditions of O_2 and 2.5 mM K Ringer). Variability between sartorii of a given frog however is probably not significant since in one experiment in which glycogen content between right and left sartorii was compared (4 frogs) the means were found to be identical. It is therefore assumed that the initial glycogen content for a given pair of muscles is the same in both muscles.

Treatment of aerobic and anaerobic muscle with 15 mM K Ringer for 90 minutes caused the level of glycogen to decline by 1.7 and 2.5 mg/g wet muscle respectively. Anaerobiosis per se caused the level of glycogen to fall by 2.0 mg/g. By dividing these values by 180 (the molecular weight of glucose) it is possible to calculate the decrement and rate of decrease in terms of glucosyl units; thus 1.7, 2.5 and 2.0 mg glycogen correspond to 9.4, 13.9 and 11.1 μmoles glucose, and the corresponding rates of glucose liberation would be 6.3, 9.3 and 7.4 μmoles glucose · grams wet muscle^{-1} · hours^{-1}.

It should be noted that the means obtained under conditions of 2.5 K/O_2 and 2.5 K/N_2 are only significantly different at a level of significance of 10%, and not at 5%, the commonly accepted value for rejection of the null hypothesis. However, it is assumed in later discussion that the mean values obtained are 'true' values and the difference between means has been used in certain calculations. Therefore it should be considered that one of the reasons for the discrepancy between the glycogen data and the heat and lactate data (noted later) is the assumption that the values found different at a level of significance of 10% are truly different.

TABLE I

Muscle Glycogen Content

2.5 K/O_2	15 K/O_2
9.15	7.26
7.78	7.05
9.46	8.08
12.62	12.27
10.03	8.41
16.14	12.27
$\bar{x}$ 10.86 $\pm$ 1.24	$\bar{x}$ 9.22 $\pm$ 0.99*

2.5 K/O_2	2.5 K/N_2
11.31	10.87
10.23	9.11
15.43	11.48
12.45	9.95
$\bar{x}$ 12.36 $\pm$ 1.12	$\bar{x}$ 10.35 $\pm$ 0.52**

(Table I cont'd . . .)

cont'd. . .

TABLE I

Muscle Glycogen Content

2.5 K/N_2	15 K/N_2
10.06	8.07
12.57	10.93
15.67	12.77
12.83	10.83
10.30	7.80
12.23	8.53
6.95	5.41
12.16	8.47
$\bar{x}$ 11.60 $\pm$ 0.90	$\bar{x}$ 9.10 $\pm$ 0.81*

All values are expressed as mg glycogen/g muscle. Means are given as $\bar{x} \pm$ SDM.

*Paired T test was performed and values found different at level of significance, $P < 0.05$.

**Paired T test was performed and values found different at level of significance, $P < 0.10$.

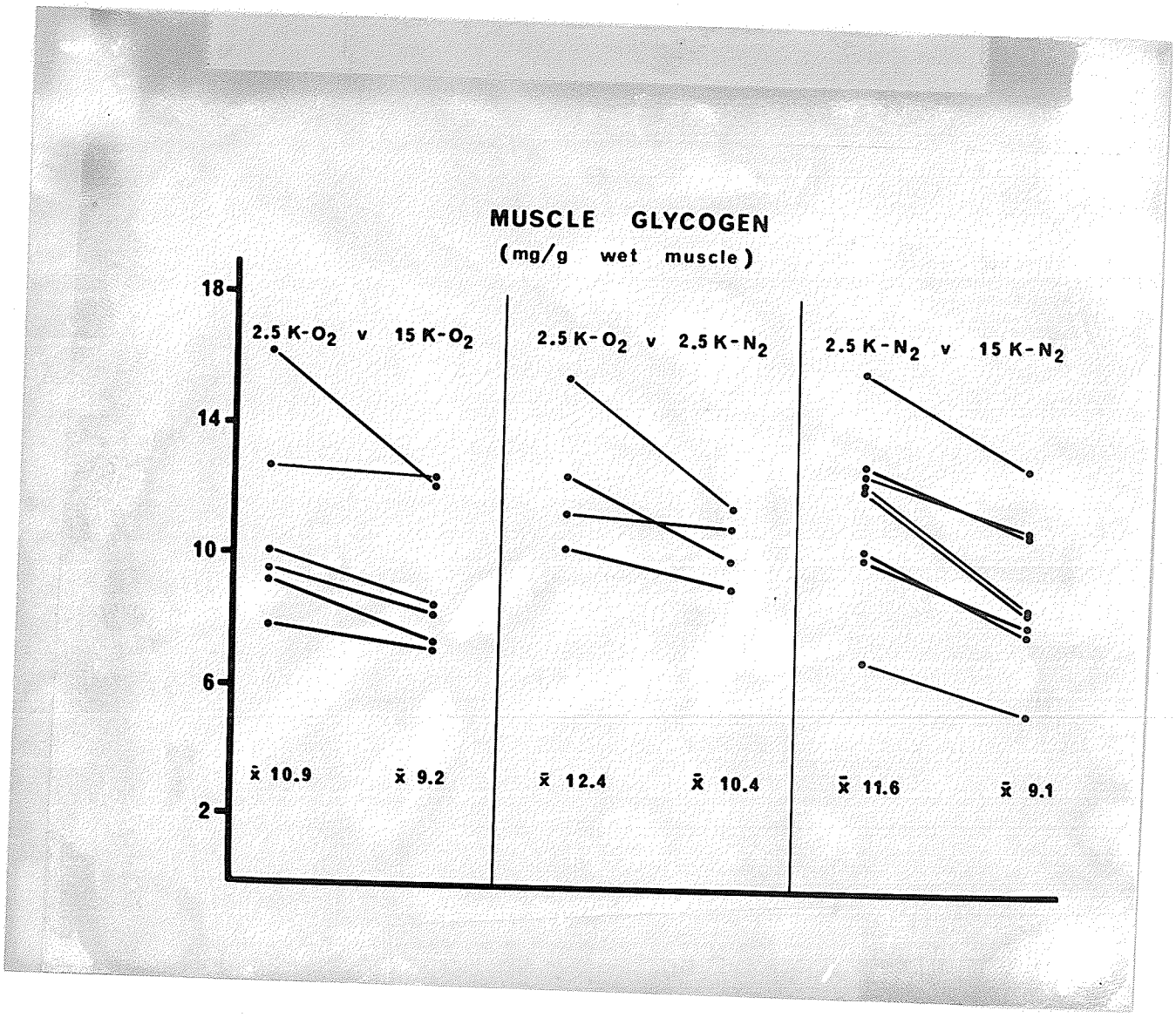


FIGURE 2. The glycogen content of the muscle (mg glycogen/g wet muscle) is plotted in control and experimental muscles as a function of the experimental conditions. Paired muscles were used, thus each set of connected points refers to the glycogen content in opposite muscles of a pair of sartorii. The experimental period was 90 minutes. The means for each condition are given at the bottom of the graph.

C. CP, ATP and ADP

The results of the measurements of the adenine nucleotides and creatine phosphate are summarized in Table II and presented graphically in Figure 3. Data for adenosine monophosphate are not presented since consistent values could not be obtained, the concentration of this metabolite apparently being too low to accurately measure by the technique outlined in Methods. The concentration of the metabolites in 'fresh' tissue (tissue isolated and frozen immediately after death) was determined for purposes of comparison. The concentrations of ATP, CP and ADP in 'fresh' tissue were 3.0, 19.1 and 0.4 μmoles per gram wet muscle respectively. Since muscle which was treated experimentally with 2.5 mM K Ringer and O_2 which supposedly approximates in vivo conditions, contained about the same amount of ATP, CP and ADP as 'fresh' muscle it was judged that the experimental procedure did not have detrimental effects on the mechanisms of ATP synthesis and utilization.

Treatment of the aerobic muscle with 15 mM K Ringer for 60 and 90 minutes caused the level of CP to fall by about a third (from 21.4 to 14.5 μmoles/g) and the levels of ATP and ADP to probably not change (3.4 and 3.2 μmoles ATP per gram and 0.4 and 0.4 μmoles ADP per gram). Treatment of the anaerobic muscle with high K Ringer for 60 minutes had similar effects; the level of CP declined from 20.2 to 12.2, ATP seemed to decrease slightly (from 4.4 to 3.7) while ADP showed no apparent change

TABLE II

Muscle Content of ATP, CP and ADP

ATP	'fresh'		2.98	3.23	3.00	3.64	2.03	$\bar{x}$ = 2.98 ± 0.27
	O_2	2.5 K_o	3.38	3.97'	3.28	2.80'	3.97'	$\bar{x}$ = 3.36 ± 0.24
		15 K_o	3.37	3.13'	3.21	2.67'	3.63'	$\bar{x}$ = 3.20 ± 0.16
	N_2	2.5 K_o	4.40	3.67	5.32	4.37		$\bar{x}$ = 4.40 ± 0.34
		15 K_o	3.08	3.35	4.55	3.97		$\bar{x}$ = 3.74 ± 0.33*
CP	'fresh'		18.24	18.63	19.78	19.81	18.82	$\bar{x}$ = 19.06 ± 0.32
	O_2	2.5 K_o	17.94'	26.07	20.08'	17.94'		$\bar{x}$ = 21.36 ± 2.43
		15 K_o	13.26'	17.58	13.33'	13.90'		$\bar{x}$ = 14.52 ± 1.03*
	N_2	2.5 K_o	24.82	20.57	17.96	17.59		$\bar{x}$ = 20.29 ± 1.67
		15 K_o	12.46	12.46	12.72	10.95		$\bar{x}$ = 12.16 ± 0.41*
ADP	'fresh'		0.39	0.40	0.49	0.47	0.44	$\bar{x}$ = 0.44 ± 0.02
	O_2	2.5 K_o	0.60	0.46	0.26			$\bar{x}$ = 0.44 ± 0.10
		15 K_o	0.66	0.34	0.30			$\bar{x}$ = 0.43 ± 0.11
	N_2	2.5 K_o	0.43	0.44	0.41	0.36		$\bar{x}$ = 0.41 ± 0.02
		15 K_o	0.42	0.62	0.39	0.35		$\bar{x}$ = 0.45 ± 0.06

All values are expressed as μmoles · g^{-1}. Means are given as $\bar{x}$ ± SDM.

'Refers to 90 minute experimental period, all other values were determined after 60 minutes.

*Paired T test was performed and values found different at level of significance, $P < 0.05$.

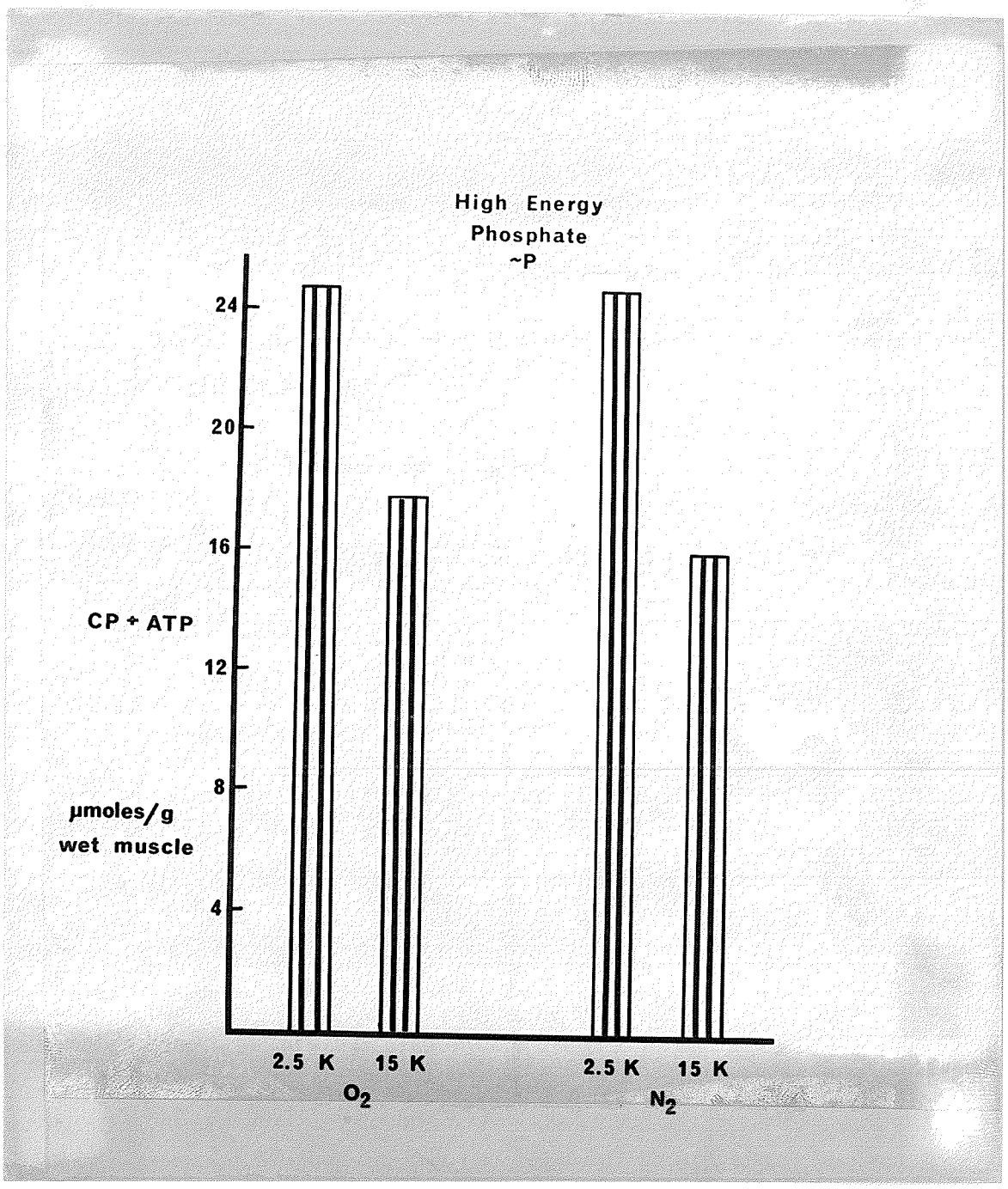


FIGURE 3. The sum of the mean content of CP and ATP (in terms of µmoles $\sim P \cdot g^{-1}$) is plotted as a function of the experimental conditions. Paired muscles were used in all experiments. Data is that presented in the preceding table.

(0.4 and 0.5 μmoles per gram respectively). In comparing the effects of treatment with 15 mM K Ringer on aerobic and anaerobic muscle, it would seem there is no apparent difference. This is especially obvious when the effects of treatment are compared in graphical form as in Figure 2 in which the sum of CP and ATP is plotted versus treatment.

It may also be concluded that anaerobiosis per se had no effect on the level of high energy phosphates, although experiments in which the effects of O_2 and N_2 in the presence of 2.5 mM K Ringer on the levels of CP and ATP were not done. The lack of effect of anaerobiosis is in contrast with 2 experiments performed on sartorii of Rana temporaria in which N_2 aeration for 90 minutes had the effect of decreasing the level of CP by about 1/3, a finding consistent with that of Ozand, Nahora and Cori, 1962.

D. Lactic Acid

The rate of lactic acid synthesis was determined for anaerobic muscle by measuring the amount of lactate in the muscle itself and the bathing solution, it being necessary for the muscle in anaerobic conditions to extrude its lactate into the extracellular medium in order to prevent muscle poisoning.

Results of measurements of the anaerobic lactate content of the Ringer solution over a two hour period are summarized in Table III and Figure 4. Resting muscle in the O_2-free environment lost lactic acid at

TABLE III

Content of Lactate in Ringer Solution

	30'	60'	90'	120'
2.5 K_o	2.54, 0, 2.73, 2.53, 3.83	0.67, 4.48, 0.54, 2.43, 5.15	5.25, 6.85, 3.92, 3.87, 4.54	4.02, 6.10, 2.68 4.21, 4.85
	$\bar{x} = 2.33 \pm 0.63$	$\bar{x} = 2.65 \pm 0.95$	$\bar{x} = 4.89 \pm 0.55$	$\bar{x} = 4.37 \pm 0.56$
15 K_o	1.21, 2.36, 0.41 3.87, 2.75	4.32, 6.20, 3.39 5.84, 6.38	7.53, 7.55, 5.17 8.73, 8.65	7.31, 15.78, 8.26 8.95, 9.56
	$\bar{x} = 2.12 \pm 0.60$	$\bar{x} = 5.22 \pm 0.59$	$\bar{x} = 7.53 \pm 0.64$	$\bar{x} = 9.97 \pm 1.50$

All values are expressed as µmoles/g wet muscle. Means are given as $\bar{x} \pm$ SDM.

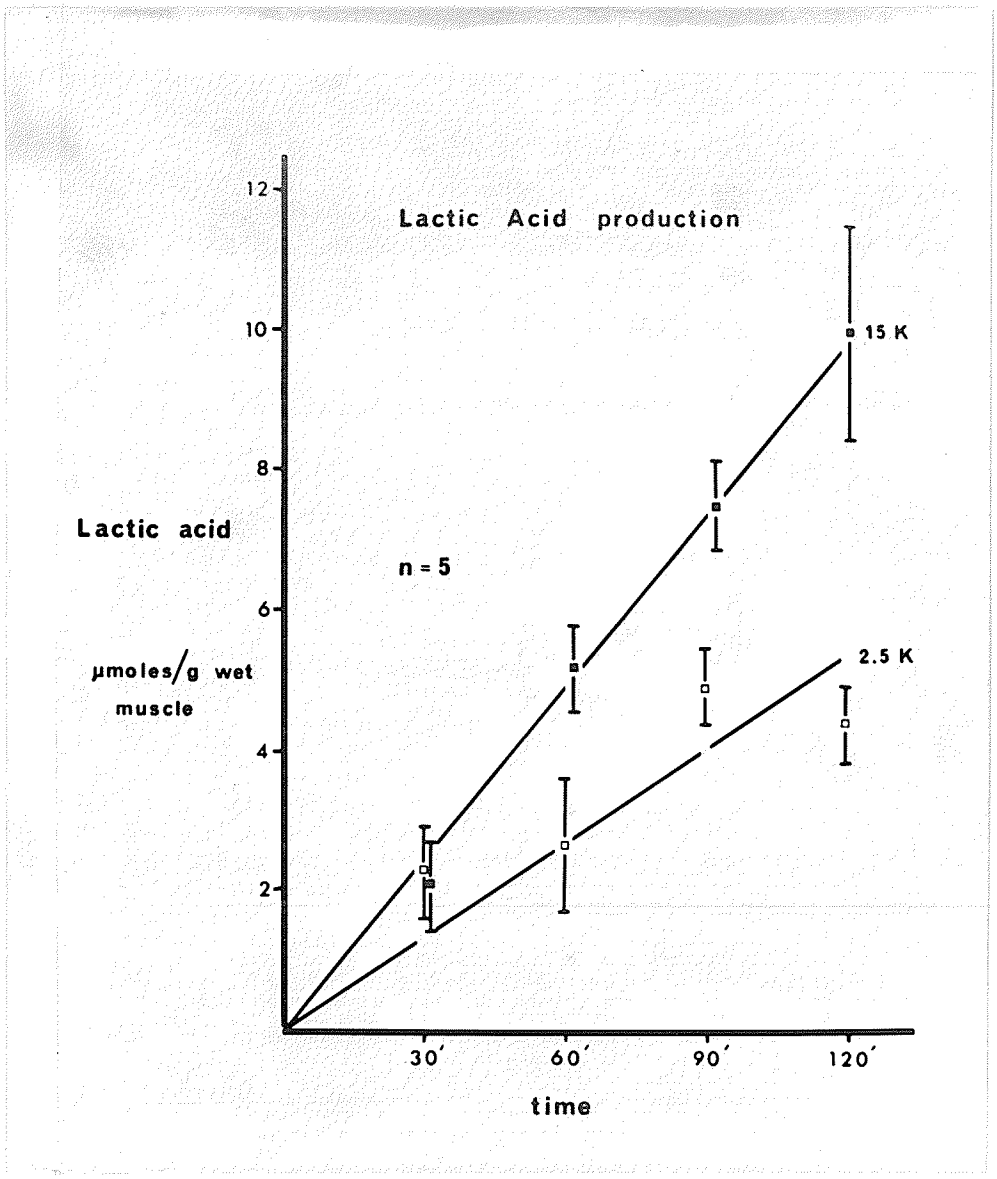


FIGURE 4. The mean ± SDM of lactic acid content of the bathing solution (Ringer) (in terms of µmoles · g^{-1}) is plotted versus the time at which the values were determined. Zero time refers to the point at which the muscles were placed in the different solutions. Each mean is the average of 5 separate determinations. Pure N_2 was bubbled through the solutions for the entire experimental period.

a rate of about 2.5 μmoles $\cdot g^{-1} \cdot hr^{-1}$. It is assumed that the rate of appearance is linear over the two hour period of measurement. Muscle treated with 15 mM K Ringer showed an increased rate of lactic acid appearance, the rate being about 5 μmoles $\cdot g^{-1} \cdot hr^{-1}$. The doubling of the rate of lactic acid 'synthesis' with depolarization is similar to the increment in heat rate (Kaye and Mommaerts, 1960, doing the same lactate determinations found the same values for the rates of production reported here).

Measurements of the intramuscular lactic acid concentration are summarized in Table IV and presented graphically in Figure 5. Aerobic muscle treated with 2.5 or 15 mM K Ringer for 60 minutes contained about 0.8 and 0.9 μmoles lactic acid per gram wet muscle respectively, while anaerobic muscle treated with 2.5 or 15 mM K Ringer contained 1.6 and 3.2 μmoles lactic acid per gram wet muscle respectively. Anaerobiosis per se had the effect of doubling the lactic acid concentration in the resting muscle. Treatment with 15 mM K_o also doubled the concentration of lactic acid in the anaerobic muscle but had little or no effect on lactic acid content of aerobic muscle.

Concerning anaerobic muscle, the rate of lactic acid synthesis may now be calculated by adding the intramuscular lactic acid to that in the bathing solution. The total lactic acid synthesized in one hour of anaerobiosis in muscle bathed in either 2.5 or 15 mM K Ringer is, therefore, 1.6 + 2.5

TABLE IV

Muscle Content of Lactate and Glucose-6-Phosphate

G-6-P		'fresh'	1.74	2.73	1.74		$\bar{x} = 2.07 \pm 0.33$
	O_2	2.5 K_o	0.03'	0.03'	0.01'	0.06'	$\bar{x} = 0.03$
		15 K_o	0.08'	0.02'	0.03'	0.06'	$\bar{x} = 0.05$
	N_2	2.5 K_o	0.01	0.02	0.05	0.03	$\bar{x} = 0.03 \pm 0.01$
		15 K_o	0.04	0.07	0.10	0.05	$\bar{x} = 0.07 \pm 0.02$
Lactate	O_2	2.5 K_o	0.88	0.73			$\bar{x} = 0.81$
		15 K_o	0.94	0.85			$\bar{x} = 0.90$
	N_2	2.5 K_o	1.35	1.83	1.45	1.70	$\bar{x} = 1.58 \pm 0.11$
		15 K_o	2.19	4.29	2.86	3.35	$\bar{x} = 3.17 \pm 0.44$*

All values are expressed as μmoles/g wet muscle. Means are given as $\bar{x} \pm$ SDM.

' 90 minute experimental period, all other values were determined after 60 minutes.

*Paired T test was performed and values were different at a level of significance of $P < 0.05$.

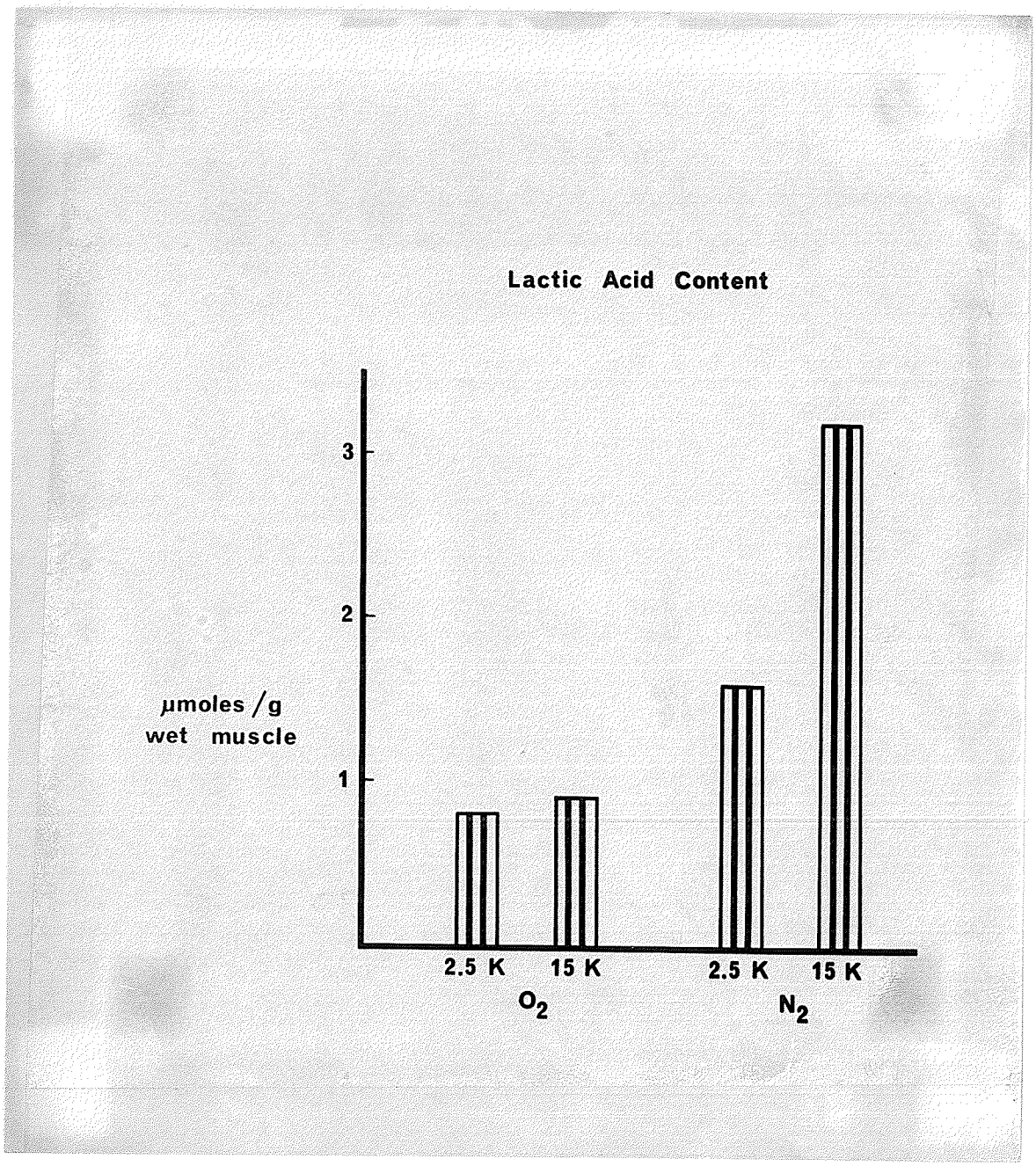


FIGURE 5. The mean lactic acid concentration of the muscle after 60 minutes (in terms of μmoles · g^{-1}) is plotted as a function of the experimental conditions. Values are obtained from the preceding table.

and 3.2 + 5.0, or 4.1 and 8.2 μmoles per gram wet muscle respectively. Since the synthesis of one molecule of lactic acid results in the synthesis of about 1.5 molecules of ATP, the rates of ATP synthesis in the above conditions are about 6 and 12 μmoles ATP per gram wet muscle per hour.

E. Glucose-6-phosphate

Measurements of the intramuscular concentration of glucose-6-phosphate are also summarized in Table IV. Data for fructose-6-phosphate is not presented since consistent values could not be obtained in muscles which had been allowed to equilibrate over a period of several hours. That is, the concentration of fructose-6-phosphate was judged to be too low to be accurately measured by the techniques as outlined in Methods. Aerobic muscle treated with either 2.5 or 15 mM Ringer contained about the same amount of glucose-6-phosphate (0.03 and 0.05 μmoles per gram wet muscle). Anaerobic muscle treated with 2.5 or 15 mM K Ringer contained 0.03 and 0.07 μmoles per gram respectively. The results suggest that treatment with 15 mM K Ringer has little, if any, effect on glucose-6-phosphate concentration in aerobic conditions, while treatment with high K Ringer in anaerobic conditions apparently caused a doubling of the concentration of glucose-6-phosphate.

The importance of allowing a period of equilibration for the determination of resting levels of glucose-6-phosphate is apparent by comparing the

results obtained with 'fresh' tissue to the experimental tissue. 'Fresh' tissue (tissue immediately frozen and analyzed after death of the frog) contained about 2 μmoles per gram wet muscle, or about 50 to 100 times the amount of glucose-6-phosphate in experimental tissue.

IV. DISCUSSION

The purpose of experiments reported here was to further investigate the marked difference between the aerobic and anaerobic increment in metabolic rate due to increased K_o which Hill and Howarth first described, and to provide biochemical data on which to attempt to provide an explanation of this difference.

A. Glycogen Estimations

Biochemical experiments performed relating to this section were done in order to compare changes in the concentration of the primary energy-producing substrate (glycogen, under the experimental conditions used here) with changes in the end product of this metabolism (lactic acid). Changes in the rate of consumption of glycogen necessarily result in changes in rate of production of ATP, the cellular mechanism of conservation of energy. Steady-state conditions in the cell necessitate a balance between ATP production and utilization, or, in other words, the rate of cellular metabolism proceeds so as to maintain a constant concentration of ATP. Non-steady state conditions will be reflected by an increase or decrease in the level of ATP (or ~P) with time. Therefore, a combination of the results of measurements of high energy phosphate levels and the results of measurements of the glycolytic substrates can be used to estimate the rates of ATP synthesis and utilization.

The first series of experiments determined the changes in levels of glycogen that occurred after treatment with 15 mM K Ringer under aerobic

and anaerobic conditions. Ninety (90) minutes after treatment with high K Ringer, the glycogen content of the muscle had decreased by 1.7 mg/g in aerobic and 2.5 mg/g in anaerobic conditions (or alternately, 9.4 and 13.9 μmoles glucose per gram, respectively). Anaerobiosis per se caused the glycogen level to decrease by 2.0 mg/g (11.1 μmoles glucose per gram) over the 90 minute period. Therefore, the total decrease in glycogen content in conditions of high K_o and N_2 can be calculated to be 2.5 + 2.0 = 4.5 mg (or 25.0 μmoles glucose) $\cdot g^{-1} \cdot 90\ min^{-1}$. The decrease in glycogen content in resting muscle in O_2 was probably negligible since no change could be detected in one experiment (theoretically it would be expected that the amount of glycogen used in aerobic resting muscle in 90 minutes would be so small as to be considered negligible).

It is possible to estimate the total ATP synthesized in the experimental period from the glycogen data since:

1) complete oxidation of one molecule of glucose to CO_2 and H_2O (aerobic conditions) can yield 38 molecules of ATP, so 9.4 μmoles glucose can yield about 360 μmoles of ATP,

2) anaerobic conversion of one molecule of glucose to lactate results in the synthesis of 3 molecules of ATP, so 25.0 μmoles glucose can yield about 75 μmoles of ATP.

Thus, assuming conditions of normal efficiency of ATP synthesis, the total ATP synthesis during treatment with 15 mM K Ringer was about 360

μmoles $\cdot$ g^{-1} $\cdot$ 90 min^{-1} in O_2 and 75 μmoles $\cdot$ g^{-1} $\cdot$ 90 min^{-1} in N_2.

The aerobic rate probably requires some adjustments; it is estimated that of the glucose released from glycogen, some 8% is released as free glucose rather than glucose-1-phosphate (Ozand, Nahara, and Cori, 1962) and that an additional 15% of the phosphorylated glucose is converted to lactate rather than oxidized to CO_2 and H_2O. If these figures are allowed for, then the rate of ATP synthesis calculated above is high by a factor of about 25%. The aerobic ATP synthesized with the above allowances would therefore be about 360 x 0.75 = 270 μmoles ATP $\cdot$ g^{-1} $\cdot$ 90 min^{-1}. Using the figures of 270 and 75 it appears that the aerobic rate of ATP synthesis is about 3.5 times the anaerobic rate during treatment with 15 mM K Ringer.

It is also possible to calculate the total ATP synthesized after 90 minutes in normal Ringer under aerobic and anaerobic conditions. Since the aerobic glycogen decrease was not determined, the ATP synthesized must be calculated from the resting heat rate, that is, from the total heat produced (or alternately, from the total O_2 consumed since it may be calculated that a resting heat rate of 2.5 mcal $\cdot$ g^{-1} $\cdot$ min^{-1} corresponds to a Q_{O_2} of 0.5 μliters $\cdot$ g^{-1} $\cdot$ min^{-1}, a value which has been obtained experimentally (Hill, 1965). This is possible since oxidation of one mole of glucose to CO_2 and H_2O under cellular conditions releases about 670,000 calories (Lehninger, 1965), or 670 mcal per μmole glucose, and in the process

synthesizes about 33 μmoles of ATP (Since it is assumed that 15% of the glucose is converted to lactate rather than CO_2 and H_2O the figure of 670,000 calories is in error. The actual quantity of heat liberated would be 0.85 x 670,000 plus 0.15 x 44,000, or about 576,000 calories per mole). Since the steady aerobic resting heat rate at 20° C is usually about 2.5 mcal · g^{-1} · min^{-1} (Hill, 1965), or 150 mcal · g^{-1} · hr^{-1}, the rate of ATP synthesis is calculated to be 33 x 150/576 = 8.6 μmoles ATP · g^{-1} · hr^{-1}, or about 13 μmoles ATP · g^{-1} · 90 min^{-1}. The anaerobic ATP synthesis can be calculated from the glycogen decrease in 2.5 K Ringer and N_2 (11.1 μmoles glucose · g^{-1} · 90 min^{-1}) by multiplying 11.1 x 3 = 33.3 and is found to be about 33 μmoles ATP · g^{-1} · 90 min^{-1}. Thus, in muscle in 2.5 K Ringer, the aerobic rate of ATP synthesis (13 μmoles · g^{-1} · 90 min^{-1}, calculated from heat data) is about one-third the anaerobic rate (33 μmoles · g^{-1} · 90 min^{-1}, calculated from glycogen data).

B. Heat Experiments

As indicated earlier, steady-state metabolic conditions represent a net balance of ATP synthesis and use. That is, over a period of time all the cellular ATP is recycled (ATP ⟶ ADP ⟶ ATP ⟶ etc.) and in the process, energy conserved in the ~P bond is released. Thus, in the steady-state all the energy that is released from glucose catabolism eventually appears as heat (glucose ⟶ 2 lactate + 44,000 calories, glucose ⟶ $6CO_2 + 6H_2O$ + 670,000 calories).

If all values for ATP synthesis are calculated from the total heat production, somewhat different values are obtained. It was determined above that the aerobic resting muscle synthesized ATP at a rate of 13 μmoles $\cdot g^{-1} \cdot 90\ min^{-1}$. Since the steady anaerobic resting heat rate is usually about 1.5 mcal $\cdot g^{-1} \cdot min^{-1}$ the ATP synthesis in 90 minutes in conditions of 2.5 K and N_2 would be $3 \times 1.5 \times 90/44 = 9.2$ μmoles $\cdot g^{-1}$. Total heat production during treatment with high K Ringer for 90 minutes may be obtained by integration of the areas of the heat curves for these experimental conditions. When this is done, it is found that some 1,000 mcal $\cdot g^{-1}$ and 220 mcal $\cdot g^{-1}$ respectively are produced in aerobic and anaerobic conditions, which would correspond to the oxidation of 1.5 μmoles glucose $\cdot g^{-1}$ to CO_2 and H_2O, and conversion of 4.9 μmoles glucose $\cdot g^{-1}$ to lactate, which in turn would correspond to a total ATP synthesis of 57 μmoles $\cdot g^{-1} \cdot 90\ min^{-1}$ aerobically and 15 μmoles $\cdot g^{-1} \cdot 90\ min^{-1}$ anaerobically.

C. Lactic Acid Estimations

In the Results section concerning lactic acid, rates of ATP synthesis were calculated for anaerobic muscle under conditions ofnormal and high K Ringer. In this case the ATP synthesis for muscle in 2.5 and 15 mM K Ringer was 6 and 12 μmoles ATP $\cdot g^{-1} \cdot hr^{-1}$ respectively.

The results of all these calculations for the rates of ATP synthesis are

summarized in Table V. It is apparent that calculations of ATP synthesis rate from glycogen data leads to much larger values than those obtained from heat or lactic acid data. It is also apparent that for anaerobic muscle the results obtained from calculation by heat and lactate data are in close agreement. Heat production and lactate are similar in terms of skeletal muscle cell biology in the sense that they are both end-products of metabolism (only in the present context of course, since lactate is still a highly complex, organic molecule and represents an 'ultimate end-product' only in skeletal muscle in anaerobic conditions). Therefore, it should probably be considered that the heat and lactate data would give the best estimation of rate of ATP synthesis, and the results obtained from the heat data will be taken as the rates of ATP synthesis in later discussion.

If the glycogen data is accurate, then it must be concluded that on the average 5 times more glycogen is released than can be accounted for in terms of end-product (heat or lactate) under all experimental conditions in which glycogen change was determined. The whereabouts of the apparent excess glucose is unknown; it is obvious from the glucose-6-phosphate data that no accumulation of hexose monophosphate occurred. It also seems unlikely that large amounts of glucose were released into the Ringer solution (in view of the figure of 8% free glucose produced from glycogen; Ozand, Nahara and Cori, 1962) although this was not checked experimentally. A possible, but highly improbable, explanation is that all the experimental

TABLE V

ATP Synthesis in 90 Minutes

	2.5 K/O_2	15 K/O_2	2.5 K/N_2	15 K/N_2
Calculated from glycogen data	-	270	33	75
Calculated from heat data	13	57	9	15
Calculated from lactate data	-	-	9	18

All values expressed as $\mu moles \cdot g^{-1}$.

muscles contained less glycogen than their control mates.

It must also be considered that the experimental results are in error. It was noted above that the change in glycogen concentration was in disagreement with the heat and lactate results by a factor of about 5 times in all cases, which would suggest that a methodological error may have occurred (for example, the standard glycogen solutions used for establishing a standard curve may consistently have been made up incorrectly). It is also known that the change in glycogen content which occurs during a short tetanic contraction (of rat skeletal muscle) can be exactly accounted for by the increase in hexose monophosphate and lactate (Cori and Cori, 1933). However, it should also be noted that the average glycogen content (about 11 mg/g) and range of values obtained of the resting muscle is similar to that obtained by others (see, for example, Danforth, 1965).

It has been well established that for several different cell types, that ATP is buffered by a larger pool of creatine phosphate (see, for example, Lehninger, 1970b) which has the effect of increasing the functional size of the ATP pool. The sum of CP and ATP has been called the 'high energy phosphate' pool and changes in the level of this is taken to represent changes in the relative rates of ATP synthesis and utilization. That is, it is assumed in the following discussion that any decline in the level of ~P is indicative of a situation in which the rate of ATP utilization is greater than the rate of synthesis. It is not clear from the experimental data whether

or not the level of ~P continues to decline after 90 minutes of treatment with high K Ringer, since later measurements were not made. The single 60 minute experiment with aerobic muscle suggests that the decline in the level of ~P may in fact plateau even before the end of the 60 minute experimental period. Results from the heat experiments suggest that muscle treated with 15 mM K Ringer assumes a new steady-state metabolism after about 45 to 60 minutes of increased K_o. It may be wiser, for the present discussion to assume that the decline in ~P seen with high K_o occurs during the first hour of treatment and that beyond this point further change may or may not occur.

Since the cellular ATP is simultaneously subject to changes in rate of synthesis and utilization, the steady-state ATP level must (as indicated previously) represent a balance between the rates of synthesis and utilization. The total ATP utilization in the experimental period may be calculated from the sum of the ATP synthesis and the decrease of the ~P pool.

The level of ~P decreased by 24.7 - 17.7 = 7.0 μmoles $\cdot g^{-1}$ in aerobic conditions, and by 24.6 - 15.9 = 8.7 μmoles $\cdot g^{-1}$ in anaerobic conditions, when muscle was treated with high K Ringer for one hour. Since 'fresh' muscle contained about the same amount of ~P as muscle treated with 2.5 mM K Ringer and O_2 or N_2, the rate of decrease of the ~P pool is taken to be zero in these conditions. Using the total ATP synthesis calculated from the heat data, the mean rates of ATP utilization can now be

calculated for the various experimental conditions as follows:

rate of ATP utilization = rate of ATP synthesis + rate of ~P decrease

The results of the calculations of mean rates of ATP utilization are summarized in Table VI.

The calculated average rates of ATP use allow some comment as to the effect of treatment with high K Ringer in aerobic and anaerobic conditions. The simplest interpretation of the data is that during partial depolarization there is increased ATP hydrolysis and that whatever is responsible for the increased ATPase activity is sensitive to the presence of O_2 (since the aerobic overall ATPase rate is double that of the anaerobic muscle during the first hour; during later periods when steady-state conditions are probably reassumed, the aerobic rate would be 3 to 4 times the anaerobic rate).

Alternate interpretations become more complex. The O_2-sensitive ATPase theory makes the assumption that oxidative phosphorylation is coupled to electron transport to the same degree in conditions of normal and high K_o (i.e. the oxidation of one mole of glucose to CO_2 and H_2O yields 33 moles of ATP, as calculated earlier). During conditions of anaerobiosis, mitochondrial function is presumably turned off because of the absence of O_2. In such conditions the rate of glucose consumption must increase several fold in order to maintain the ATP level; that this does in fact happen is suggested by the results obtained in this study in which it has

TABLE VI

Mean Rates of ATP Utilization

Conditions	Total ATP synthesis in 1 hour	+	~P decrease in 1 hour	=	ATP utilization in 1 hour
2.5 K/O_2	8		0		8
15 K/O_2	38		7		45
2.5 K/N_2	6		0		6
15 K/N_2	10		9		19

All values expressed as $\mu moles \cdot g^{-1} \cdot hr^{-1}$.

been calculated that the rate of ATP synthesis is approximately the same in resting muscle in the presence of either O_2 or N_2. Treatment of the anaerobic muscle with 15 mM K Ringer caused the level of ~P to decrease by about one-third and the level of glycogen to fall by 2.5 mg/g, suggesting that in conditions of no mitochondrial function, the rate of ATP synthesis via increased glycolysis is not quite sufficient to make up the ATP lost through hydrolysis (at least during the first hour of depolarization). Therefore, if it were true that sustained partial depolarization were to cause disruption of the process of oxidative phosphorylation in aerobic conditions, it would perhaps be expected that the muscle would react as though it were faced with anaerobic conditions; that is, the glycolytic rate would probably increase and the level of ~P perhaps fall. The results indicate that in aerobic conditions and sustained partial depolarization (treatment with 15 mM K Ringer) there is stimulation of glycogenolysis (1.7 mg/g · 90 min) and decreased levels of ~P (7.0 μmoles/g · 60 min), stimulation of lactate synthesis was not seen however but this can perhaps be explained by the fact that normal oxidation of the glycolytic products can still occur in the mitochondria. In other words, if treatment with high K Ringer were to disrupt the mitochondrial function of ATP synthesis, the true rate of ATP synthesis would be determined in the same manner as for anaerobic metabolism (that is, each μmole of glucose produced from glycogen would synthesize 3 μmoles of ATP) and the calculated rate of ATP synthesis would be 9.4 x 3 =

28.2 μmoles ATP · g^{-1} · 90 min^{-1}, or about 19 μmoles ATP · g^{-1} · hr^{-1}. And if the glycogen data can be assumed to be in error by a factor of 5, the rate of synthesis would be about 19/5 ≈ 4 μmoles ATP · g^{-1} · hr^{-1}, which when combined with the decrease in ~P would give a mean rate of ATPase activity of 4 + 7 = 11 μmoles · g^{-1} · hr^{-1} (in contrast to the mean rate of 45 μmoles · g · hr^{-1} calculated by assuming normal mitochondrial function).

It may also be true that the process of oxidative phosphorylation is impaired during aerobic partial depolarization but not completely obliterated. It was noted that the mean rate of ATP use in aerobic and anaerobic resting muscle was about the same; if it were true that partial depolarization induced an increased rate of ATP hydrolysis to the same degree in aerobic and anaerobic muscle, then, in this case, the true rate of ATPase activity would be about 10 μmoles · g^{-1} · hr^{-1} in both cases (i.e. the rate in 15 K/N_2). It was calculated that partially depolarized aerobic muscle with zero mitochondrial ATP synthesis could synthesize ATP at a rate of 4 μmoles · g^{-1} · hr^{-1}, while with 'normal' mitochondrial function the rate of synthesis would be 38 μmoles · g^{-1} · hr^{-1}. Thus, if the true rate of ATP synthesis were 19 - 7 (the decrease in ~P · hr^{-1} in 15 K/O_2) = 12 μmoles · g^{-1} · hr^{-1}, then there is suggestion that the process of oxidative phosphorylation was incompletely impaired during treatment with high K Ringer.

It has been argued in the previous section that treatment of aerobic

muscle with 15 mM K Ringer interferes with the process of oxidative phosphorylation by mitochondria. If this is true, then some link between depolarization of muscle membrane and mitochondria must exist. As noted in the Introduction, several authors have concluded that partial depolarization releases bound Ca^{++} ions into the sarcoplasm (in the same way as probably occurs during normal excitation-contraction coupling). It has also been established that the energy released by mitochondrial oxidation may be utilized for purposes of ion translocation, and that in certain conditions ion translocation may occur in preference to oxidative phosphorylation (see Lehninger, 1970a). In such conditions the apparent efficiency of ATP synthesis would be decreased. In other words, a possible link between membrane depolarization and mitochondrial function are Ca^{++} ions. An objection to this theory is that relatively massive amounts of Ca^{++} are required for uncoupling to occur, at least in isolated mitochondria, and that since muscular contraction does not occur with partial depolarization, the free Ca^{++} level cannot rise above the mechanical threshold level of 10^{-7} M.

D. Biophysics v Biochemistry

The results of heat experiments performed by Hill and Howarth, and in the present study indicated that treatment of muscle with high K Ringer caused a different degree of metabolic stimulation in oxygenated and oxygen-free muscle (aerobic stimulation being greater than anaerobic). The results

of the biochemical determinations performed in this study have demonstrated that anaerobic muscle treated with 15 mM K Ringer probably shows a doubling of metabolic rate, while the steady-state aerobic stimulation was probably 2 to 4 times greater. Thus, it was demonstrated biochemically that the metabolic increment due to high K_o was apparently greater in aerobic than in anaerobic muscle.

E. Species Variability

Every author investigating the effect of increased K_o on muscle metabolism has indicated that an increased metabolic rate occurs (as measured by heat production or O_2 consumption). However, no author has attempted any sort of comparative study of the effect in different animal (amphibian) species. When the data for the degree of metabolic stimulation in different species is compiled as in Table VII, some interesting conclusions can be made.

(a) The effect of increased K_o on metabolism is species specific; the ratio of stimulated to basal metabolic rate is consistently greater in Rana temporaria, as compared to either Rana pipiens or Bufo marinus. Data indicates that 20 mM K_o caused an increase in $\dot{h}$ in R. temporaria of about 15 to 20 times, while the same K_o in R. pipiens caused a 2.5 to 5 fold increase.

(b) The increment in metabolism is apparently different if Q_{O_2} is compared with $\dot{h}$. R. temporaria, which had a maximum increment in heat

TABLE VII

Data for Metabolic Rate

Species	BMR	Standardized BMR	Stimulus	Stimulated MR	Stimulated: Basal Ratio	Reference
R. temporaria	$\dot{h} = 2$ mcal · g^{-1} · min^{-1}	8×10^4 ergs · g^{-1} · min^{-1}	19 - 20 mM KCl	26.4 - 64.3	13 - 32 : 1	Yamada, 1970
R. temporaria	-	8	18 mM KCl	70 - 180 ergs · g^{-1} · min^{-1}	8.8 - 22.5 : 1	Hill and Howarth, 1957
R. temporaria	$\dot{h} = 2.6$ mcal · g^{-1} · min^{-1}	10	15 mM KCl	16	6 : 1	Clinch, 1968
R. temporaria	$Q_{O_2} = 58$ µl · g^{-1} · hr^{-1}	16	20 mM KCl	409	7 : 1	Novotny et al., 1966
R. temporaria	$Q_{O_2} = 77$ µl · g^{-1} · hr^{-1}	19	18 mM KCl 15.5 mM KCl	491 - 677 429	6.4 - 8.8 : 1 5.6 : 1	Novotny et al., 1962
R. pipiens	$\dot{h} = 5.9$ mcal · g^{-1} · min^{-1}	19	16 mM KCl	15	2.5 : 1	Smith and Solandt, 1938
R. pipiens	$\dot{h} = 2$ mcal · g^{-1} · min^{-1}	8	15 mM KCl	12	6 : 1	Borowski, 1972
R. pipiens	$Q_{O_2} = 0.5$ µl · g^{-1} · min^{-1}	10	20 mM KCl	2.5	5 : 1	Hegnauer et al., 1934
R. pipiens	$Q_{O_2} = 4.5$ µmoles · g^{-1} · hr^{-1}	25	20 mM KCl	16	3.8 : 1	Van der Kloot, 1967
B. marinus	$Q_{O_2} = 0.206$ µl · mg^{-1} · 4 hr^{-1}	13	15 mM KCl	0.515	2.5 : 1	Muller et al., 1960

All values of standardized BMR are given as 10^4 ergs · g^{-1} · min^{-1}. All values of stimulated MR are given in the same units as the BMR.

rate of at least 30 times showed only a 9 times increase at most in Q_{O_2}.

Novotny et al. (1962) suggested that the 9 fold increment in Q_{O_2} observed by themselves was very near to the maximum rate of O_2 consumption of the muscle (perhaps within about 20%). If this is so, then one has to wonder how a sustained 30 fold increment in heat rate can occur. One explanation is as follows; it can be calculated from the equation:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O, \quad \Delta H = 670{,}000 \text{ calories}$$

that a heat rate of 2.5 mcal $\cdot g^{-1} \cdot min^{-1}$ corresponds to a rate of O_2 consumption of 0.5 $\mu l \cdot g^{-1} \cdot min^{-1}$. Thus the apparent resting Q_{O_2} of Novotny would correspond to a rate of heat production of 5 mcal $\cdot g^{-1} \cdot min^{-1}$, or about 2 1/2 times the rate reported by Hill and Howarth (1957) and Yamada (1970). If the true resting rate of metabolism is closer to the figure estimated by measurements of heat production, then the basal Q_{O_2} would perhaps be closer to about 30 $\mu l \cdot g^{-1} \cdot hr^{-1}$ and the ratio of stimulated to basal metabolic rate would approach a value of 20, which is perhaps an average figure for the increment in $\dot{h}$ seen in R. temporaria.

It was argued previously that the increase in heat rate and O_2 consumption seen with 10 to 20 mM K_o might represent a condition of impaired efficiency of mitochondrial ATP synthesis. It has just previously been shown that the same level of K_o caused a different degree of metabolic stimulation in frogs of different species. If the conclusion concerning impaired mitochondrial function were true, then the species variability would

suggest that different species of frogs showed a different degree of mitochondrial impairment given the same K_o (between 10 to 20 mM). Two experiments performed at the end of the study suggest that the postulated species specific mitochondrial sensitivity may exist. Using sartorii from R. pipiens, the resting heat rate and stimulated heat rate (with 15 mM K_o) were determined. The muscles were then returned to normal Ringer and 2,4-dinitrophenol (2,4-DNP) was added in a step-wise fashion until the stimulation in heat rate was about equal to that of 15 mM K Ringer (which required 15 μM 2,4-DNP). In the next experiment, a pair of sartorii from R. temporaria were set up and treated sequentially with normal Ringer, high K Ringer, normal Ringer and normal Ringer plus 15 μM 2,4-DNP. High K Ringer and 15 μM 2,4-DNP both caused the heat rate in R. pipiens to triple, while similar treatment in R. temporaria caused the heat rate to increase by 12 1/2 and 8 times respectively. This data indicates that muscle which apparently shows a greater degree of metabolic stimulation due to partial depolarization, also shows a greater degree of sensitivity to 2,4-DNP (a known uncoupler of oxidative phosphorylation).

F. Glycogen as Muscle Cell Fuel

It is apparent that since an exogenous supply of oxidizable substrate was not present in any of the experiments the muscles must have relied on endogenous fuel stores. In frog sartorius muscle (a white, twitch or phasic type muscle) it seems that glucose (stored in polymeric form as glycogen) is the

primary substrate for energy-producing metabolism since:

1) electron microscopy reveals large, diffuse deposits of glycogen

2) changes in the concentration of glycogen are sufficient to explain the metabolic rate observed experimentally

3) frog sartorius muscle has a high glycolytic capactiy; that is, the concentrations of glycolytic enzymes are relatively high and the glycolytic rate is very sensitive to various external stimuli (such as depolarization with high K_o, treatment with caffeine, electrical stimulation, and treatment with epinephrine and insulin (Ozand and Nahara, 1964; Karpatkin, Helmreich, and Cori, 1964)).

V. CONCLUSIONS

1) Treatment of frog skeletal muscle with 15 mM K Ringer causes an increase in metabolic rate as determined by either heat production or changes in biochemical substrates.

2) The change in metabolic rate is apparently O_2-sensitive since the anaerobic increment is less than the aerobic increment, as measured by heat production (or the calculated overall rate of ATP hydrolysis). The original observation of Hill and Howarth regarding this differential metabolic stimulation has, therefore, been confirmed.

3) The theory is advanced that the stimulation of metabolism seen with 10 to 20 mM K Ringer in aerobic conditions is indicative of an impairment of mitochondrial function, specifically, a partial uncoupling of oxidative phosphorylation.

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