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ABSTRACT

A system was developed to measure tension, efflux of lactate and pyruvate and acid base exchange in rat diaphragm strips. Changes in measured values for these variables were made in response to anoxia, increase in muscle activity and changes in the CO_2 level and bicarbonate concentration.

An increase in pCO_2 has a stimulating effect on the maximal tetanus tension while a decrease in bicarbonate concentration depressed isometric tension.

There is a steady lactate efflux of approximately 120 nM/g per minute during resting conditions which can reach a maximum of 1400 n M/g per minute during anoxia. Pyruvate is about 10% that of the lactate efflux in the resting state. An increase in extracellular pH (due to changes in pCO_2 and HCO_3^- concentration) results in a 20 fold increase in lactate efflux.

There is a steady net acid efflux in the order

of 250 nM/g per minute during control conditions. The net acid efflux increases following a decrease in pCO_2 and decreases or reverses in response to an increase in pCO_2. A decrease in bicarbonate concentration has an effect similar to that of an increase in pCO_2. Assuming that the changes in acid base exchange in response to changes in pCO_2 are due to passive HCO_3^- movements then it can be shown that a large fraction of the HCO_3^- generated within the muscle fibres by non-bicarbonate buffers is transferred within one hour following a change in pCO_2. It is possible to calculate an apparent permeability coefficient for bicarbonate ($\bar{P}_{HCO_3^-}$) which varies under different conditions from a value of 0.8×10^{-7} cm/sec to 2.62×10^{-7} cm/sec.

CHAPTER I

Introduction

There are three basic factors involved in acid base balance in muscle cells. They are: metabolism, (production of lactic acid mainly), buffering, and transmembrane movements of acids and bases.

Consideration of the role of metabolism in acid base balance would include asking questions such as: to what extent does lactate accumulate in muscles under different conditions? What effect does this lactate accumulation have on metabolism and the function of the fibre and how is it eliminated from the cell? What is important with respect to acid base balance is at what rate, and in what form does lactate cross the membrane? The question of whether transmembrane movements of lactate are in the ionic or the undissociated acid form is especially critical when considering the maintenance of intracellular pH. Transmembrane movement of the lactate ion only would necessitate

some mechanism(s) to compensate for the hydrogen ions remaining in the cell.

In order to discuss muscle buffering and transmembrane movements of acids and bases it is important to answer the questions: what methods are used to determine intracellular pH and how reliable are they? Having established the most reliable methods of intracellular pH measurements it is then possible to deal with the particulars of acid base balance for the muscle cell. Such things as muscle buffer value, effects of changes in pCO_2 and external bicarbonate concentration and membrane permeability to bicarbonate ions will be considered as three areas of major significance in muscle acid base balance.

A. Lactic Acid

1) Historical Research on Lactic Acid in Muscle

Acid formation was first discovered in the muscles of hunted stags by Berzelius in 1807. He was able to demonstrate that this acid was the same as that from milk. However, it was not until 1907 that Fletcher and Hopkins made accurate measurements of lactic acid in

frog muscles. At this time they showed that previous methods of facilitating extraction such as chopping of the muscle, treating with alcohol, chloroform or boiling water caused an immediate increase in the production of lactic acid. They obtained an average figure for the lactate yield of resting muscle of between 0.02% and 0.035% of muscle weight (i.e. 2.2 - 3.9 uM/g). The resting lactate value for mammalian muscles given by Fletcher in his 1913-1914 paper is 5.2 uM/g.

In the 1907 paper, Fletcher and Hopkins also established the relationship between fatigue and lactate increase, reaching a maximum in their preparation of 14.0 uM/g. Anoxia was found to increase lactate levels to between 40-50 uM/g. However, work by Hill and Kupalov (1930) indicated that during anaerobic excitation of frog muscle contraction ceases at 0.3% (33.3 uM/g) lactic acid. Two factors limiting activity for muscle in vitro were O_2 supply and the presence of carbohydrate. These limitations could be circumvented by use of thin muscle in fully oxygenated solution and the addition of glucose to the medium.

Eggleton, Eggleton and Hill (1928) calculated a coefficient of diffusion for lactic acid following fatigue in frog muscle. This was shown to be dependent on the state of fatigue, in that a higher level of lactate in the muscle due to increased stimulation resulted in a lower coefficient of diffusion. This they proposed to be due to a swelling of the muscle fibres which, in turn, reduced the extracellular space in which diffusion is more rapid, resulting in an overall decrease.

Margaria, Edwards and Dill (1933) obtained results for the rate of disappearance of lactic acid in the blood of humans after exercise. Comparing these results with the rate of removal of the total amount of lactic acid in the body of mice, after exercise, gave evidence that lactate is freely diffusable between muscles and blood (Margaria and Edwards, 1934).

Other early investigators attempted to correlate measured lactic acid changes in blood with pH changes during violent bouts of exercise (Barr, Himwich and Gree, 1923; Bock et al, 1928).

However, direct measurements of

pH changes were not taken until the work of Lang (1934). His work on humans showed fairly close agreement between the measured pH (glass electrode) of venous blood and the pH calculated from the rise in lactic acid in the blood.

2) Resting Lactate Efflux in Rat Diaphragm

Some of the earliest measurements of lactate efflux were made on incubated preparations in which whole or hemidiaphragms were placed in Warburg flasks for a period of time. Most values obtained were similar. Examples are Walaas and Walaas (1950) 104 nM/g per minute, Beatty et al (1960) 80 nM/g per minute and Peterson et al (1961) 148 nM/g per minute. These values for incubated preparations, however, did not agree with those of Hollanders (1968) and Rowlands (1969 a and b) for perfused and Bülbring type (isolated phrenic nerve and diaphragm) preparations. They obtained values closer to 300 nM/g per minute. Rowlands also in her second paper in 1969 sampled incubated diaphragms and obtained values betwen 45 and 110 nM/g per minute.

She attributed this difference to the rise in lactate concentration of the bathing solution in the incubated preparations, which in turn depressed the equilibrium reaction of lactate production. However, it must be pointed out that Kratzing (1952), using a similarly incubated preparation, obtained lactate efflux values of 440 nM/g per minute.

All the pefused and Bulbring preparations (Hollander 1968, Rowlands 1969 a and b) showed an initial high efflux of lactic acid (500-700 nM/g per minute) which gradually decreased 30 minutes after the start of each experiment to the steady resting level of 300 nM/g per minute. Hollanders (1968) suggested that this was due either to anoxia produced during the preparation of the tissue, release of adrenaline from handling the rat before killing, or slow release of bound insulin. However, Rowlands (1969 b) giving arguments against these three prosals, felt that the high initial lactate output even in glucose-free medium, was an inevitable event in the preparation and was derived from muscle glycogen.

Two factors affecting lactate production were shown by the work of Hansen et al (1951). In incubated diaphragms, lactate production was greater with muscles from rats fed a high fat diet compared with rats fed a high carbohydrate diet. Also, if the rats were fasted for 24 hours before being sacrificed, the lactate formation was greater. Lactate production was also shown to be greater in male versus female and decreased by low temperatures (Rowlands 1969).

3) Maximum Concentration of Lactate in Muscle

Two major sources of stimuli for increases in lactate production are anoxia and increased activity. However, it is significant to note that lactic acid does not increase during activity if the energy expended can be met by oxidative processes (Margaria, Edwards and Dill, 1933 and Margaria et al, 1963). According to these investigations, if activity is elevated above the level corresponding to maximum oxygen consumption, then lactic acid

should be increased at a rate proportional to the intensity of the exercise. This hypothesis has, however, been questioned (Huckabee 1958). There does seem to exist an upper limit to the concentration at which lactic acid can accumulate in the muscle. For isolated frog muscle this concentration was 14 uM/g during increasing stimulation (Fletcher and Hopkins 1907). This value is low compared with the results of Mainwood, Worsley-Brown and Patterson (1972) in which lactate concentrations of isolated superfused frog sartorius reached 20-25 uM/g during fatiguing contractions in high bicarbonate Ringer solutions. Interestingly, these values for frog sartorius compare favourably with maximal lactate concentrations of 20-25 uM/g from human muscle biopsies, depending upon the level and duration of work (Diamant et al 1968, Karlsson 1971, Edwards et al 1977 and Karlsson et al 1975).

4) Maximal Rates of Lactate Efflux

Maximal rates of efflux are ill-defined and

difficult to interpret from the literature due to the difference in preparations and methods of obtaining increased activity. Hollanders (1958) using perfused rat diaphragm, obtained a tetanic contraction by supramaximal stimuli at 50/sec which resulted in a maximal lactate efflux of 1-8 uM/g per minute. This is high compared to the perfused gastrocnemius muscle of the dog which was subjected to supramaximal tetanic stimulation of the nerve at 20 per minute for 7 minutes. Maximal lactate output was 0.6 uM/g per minute (Di Prampero et al 1970). Superfused frog muscles after a fatigue for 200 seconds at 2 tetani per second by direct stimulation reached levels of between 0.3 and 0.4 uM/g per minute, depending on the type and concentration of external buffer being used.

Results for humans are presented by Margaria et al (1964) by having subjects run to exhaustion on a treadmill in less than 40 seconds. Their maximal rate of lactate efflux was 1.6 uM/g body weight per minute which would be much

higher if corrected for only those tissues responsible for the increase in lactate.

5) Mechanism of Lactate Efflux

There are two stages which must occur before lactate efflux can take place. The first is a passage through the membrane which can be by a carrier mechanism which would be rate limited with kinetics similar to enzyme action, transport through pores in the membrane or carried in the lipid phase. The second stage is diffusion through the extracellular space. This would vary with tissue thickness, effectiveness of perfusion, diffusion coefficients, etc.

As mentioned previously, one of the earliest mechanisms proposed to limit lactate efflux was that of Eggleton et al (1928) in which swelling of the fibres was postulated to reduce the extracellular space, thereby enhancing the limiting effect of diffusion across the membrane. Another proposal was a saturation type of efflux mechanism from measurement on frog sar-

torius (Karpatkin et al 1964). These experimenters obtained a V_{max} of 0.8 uM/ml of intracellular water per minute.

Many results have shown that the rate of lactate efflux is limited by something other than simple diffusion. Hirche et al (1970) and Karlsson et al (1971) demonstrated this in dog gastrocnemius and Mainwood and Worsley-Brown (1975) in frog sartorius. Kubler et al (1966) postulated that an active transport might be involved.

If passage through the membrane is the only limiting stage to the rate of lactate efflux, exactly what mechanism is operative will depend largely upon the form in which the substance is transported across the membrane, (i.e. as the lactate ion or the lactic acid molecule).

6) Transport Across the Membrane

a) Transmembrane Movements of the Lactic Acid Molecule

If lactate is leaving the fibre as the lactic

acid molecule, then there should be a concurrent fall in pH in the extracellular medium. Work by Dubuisson (1934) showed a fall in pH of the bicarbonate buffer on the surfaces of muscles immediately after activity. Since the results were obtained by glass electrodes on the surface of the muscles, then production of acid in the cell would react with intracellular bicarbonate and liberate equivalent quantities of CO_2 which would diffuse from the muscle to the exterior. Therefore, the CO_2 trapped at the surface by the electrode would result in a change in pH recordings whether or not the protons are confined to the fibre. A larger change would be expected if they were transported across the membrane.

Another approach to provide evidence for the transport of the lactic acid molecule is the results of measurements of shifts in the CO_2 dissociation curves of human blood following severe exercise (Hill et al 1924; Bock et al 1927). These were interpreted as being due to all the lactate in the blood having reached it as lactic acid from the muscle

(Henderson 1928; Turrell and Robinson 1942).

Roos (1975) performed experiments on incubated rat diaphragms in which he measured the D-lactate distribution ratio and intracellular pH after exposure to solutions buffered to a broad range of pH values and containing varying concentrations of labelled D-lactate and potassium. He also performed studies to determine the length of incubation necessary to reach steady distribution. The relation between the steady state ratio of total intracellular to total extracellular lactate and the ratio of hydrogen ions from outside to inside the cell indicated that it is predominantly the undissociated lactic acid molecules, rather than the much more numerous lactate ions which permeate the fibre membrane. This was further supported by membrane depolarization with potassium which did not significantly alter this relation.

Mainwood and Worsley-Brown (1975), working with superfused frog sartorius, also showed that the efflux of undissociated acid can make a considerable contribution to net lactate efflux but only when

external pH and buffer concentration are high. This conclusion was based on two observations. First, membrane depolarization with potassium at high external buffer concentrations had very little effect on lactate efflux. Secondly, the ratio of lactate to hydrogen ion efflux decreased as buffer concentration was raised.

b) Transmembrane Movements of the Lactate Ion

At an intracellular pH of 7.0 and with a pK of the lactate-lactic acid system of 3.7 (Lockwood, Yoder and Zienty, 1965), the lactate ion is about 2,000 times the concentration of the molecule. The question arises: is the lactic acid molecule permeability relative to that of the lactate ion great enough to account for the measured efflux of lactate in spite of the greater abundance of the ionic form?

Woodbury and Miles (1973) using frog skeletal muscle measured resistance and transmembrane potential after replacing one anion by another in the

bathing medium. Thus, they were able to show that lactate has a low but finite permeability which is approximately 0.07 that of chloride.

More direct evidence for the movement of lactate ions was presented by Mainwood and Worsley-Brown (1975). In low buffer solution (1 mM), depolarization with isotonic potassium sulphate resulted in a pronounced decrease in lactate efflux. This, they concluded, was an indication of lactate ions being responsible for the greater part of the efflux. Other evidence was a high ratio of lactate to hydrogen ion efflux in low buffer concentrations.

7) Effect of pH on Lactate Efflux Results

There are at least two ways in which pH appears to be able to affect lactate efflux results. At the intracellular level, a decrease in pH can result in a slowing of glycolysis due to the sensitivity of rate limiting phosphofructokinase (PFK) reaction to the hydrogen ion concentration (Mansour 1963; Danforth 1965; Trivedi and Danforth 1966). The

slowing of glycolysis could result in a decreased lactate production giving rise, in turn, to lower measured lactate efflux.

A second possibility is seen in the results which indicate that a relationship exists between external pH and anion conductance, including lactate (Hutter and Warner 1967a and b; Woodbury and Miles 1973).

Harken (1975) altered the external pH of the isolated canine hindlimb between 6.9 and 7.6. He showed that lactate production was linearly related to pH. This may have been due to changes in intracellular pH or the PFK system or, perhaps, by increasing the efflux of the lactate ion, the lactate pyruvate equilibrium was altered. In isolated frog sartorius an increase in pH has been shown to cause an increase in lactate efflux (Mainwood et al 1972; Mainwood and Worsley-Brown, 1975).

B. Buffering and Transmembrane Movements of Acids and Bases

1) Early Measurements of Intracellular pH

The picture of acid base balance in muscle fibres is not very clear. Historically, the problems centered around obtaining an accurate measurement of intracellular pH (pH_i). The typical means of measuring pH_i for muscle fibres have been measurement of the pH of a tissue brei; measurements on the colours of pH indicators; measurements on the distribution of weak acids or bases and measurements using microelectrode systems (Caldwell 1956).

Early measurements of pH_i obtained by these methods have been made by various investigators. For example, Turusawa and Kerridge (1927a), using measurements with a glass electrode on tissue brei of cat gastrocnemius at 0°C, obtained resting values for pH_i of 7.04. Adjusted for temperature, this became a pH_i of 6.90. This was reduced to 6.26 and 6.02 during fatigued and rigor conditions respectively. The first use of a weak acid method to determine pH_i of muscle was performed by Fenn (1928) on frog gastrocnemius. He obtained resting

values of pH_i of 7.2 to 7.3 at a CO_2 tension of 20 mmHg. His fatigued and rigor results were 6.6 and 6.8 respectively. With a similar method on frog sartorius Stella (1929) obtained a pH_i of 6.9 for the same CO_2 tension. However, there was the possibility of lactic acid accumulation during Stella's procedure which would result in a true resting pH_i higher than what was calculated.

The first reliable measurements of pH_i with microelectrodes were made by Caldwell (1954a, 1954b, 1956) using a glass microelectrode. However, with microelectrodes one is limited to large cells only. He was able to obtain a resting pH_i of 6.9 for crab muscle fibres. Contractures initiated with 0.6 M KCl lowered the pH_i by less than 0.1 of a pH unit.

2) Maintenance of Intracellular pH

These results and others (Caldwell 1956) showed the general agreement that can be obtained by different methods of measurement. What, then, one may ask are the factors giving rise to the observed

pH_i? In his original paper, Donnan (1911) implied that the hydrogen ions inside the cell are in a Donnan equilibrium with those outside. If both potassium and hydrogen ions obey the Donnan relationship then the equation

$$\frac{[H^+]\text{ extracellular}}{[H^+]\text{ intracellular}} = \frac{[K^+]\text{ extracellular}}{[K^+]\text{ intracellular}}$$

should hold. Increased evidence for the credibility of this equation was given by Mond and Netter (1930), Boyle and Conway (1941) and Conway and Fearon (1944). Boyle and Conway (1941) supported the above equation quoting the pH_i value of 5.6 obtained by Rous (1925) using intracellular indicators on mouse muscle. However, the results of Rous were criticized by Fenn and Mauren (1935) on two counts. First, the indicators he used did not penetrate into cells and the values therefore were for the extracellular space. Secondly, the techniques involved damage to the tissue which has been shown to have a more acid reaction to indicators (Chambers et al 1927, Fenn and Mauren 1935).

Measurements of pH_i using the carbon dioxide/

bicarbonate method have indicated that postulation of a Donnan equilibrium is false. The results give pH_i values much higher than would be expected from a Donnan equilibrium (Fenn 1936; Stella 1929; Hill and Kupalow, 1930; Hill 1931).

The criticism by Conway (1941) and Conway and Fearon (1944) of the use of CO_2 distribution to determine pH_i centered around the fact that much of the acid-labile carbon dioxide of muscle is not bicarbonate. Therefore, another substance was sought to be used in lieu of CO_2. One such substance was 5.5-dimethyl-2,4-oxazolidinedione (DMO) used by Waddell and Butler (1959) for determination of pH_i on dog muscle. They obtained an average pH_i of 7.04 which agrees with the results of many others using the CO_2 distribution methods on mammalian tissues (Tobin 1956; Nichols 1958). This technique was used shortly thereafter on rat diaphragm in vitro by Miller, Tyson and Relman (1963) to obtain a pH_i of 6.89.

Except for the work of Carter et al (1967a and b), in which pH sensitive glass microelectrodes were used in rat skeletal muscle, almost all measure-

ments have supported a pH_i that is too high to be maintained by a Donnan equilibrium. However, Paillard (1972) has clearly shown that the type of microelectrode used by Carter et al had portions of the pH sensitive tip exposed to the extracellular space. The more recent results of Hinke and Menard (1976) using pH microelectrodes provide clear evidence that the H^+ ion never reached a Donnan equilibrium across the membrane even in fibres which were exposed from 3 to 5 hours to normal Ringer solution. These investigators also conducted an excellent comparison of the DMO and microelectrode methods using barnacle muscle fibres. The DMO-pH and the electrode pH_i compared favourably, provided the DMO-pH_i was calculated by extrapolating the slow uptake phase of the indicator compounds (^{14}C-DMO and ^{3}H-insulin) to time zero.

Other evidence has come from the work of Hill (1955) on lactic acid production of frog sartorius. Using phosphate buffers, he lowered the extracellular pH (pH_e) to 6.0 and observed its effect on the formation of lactic acid in response to stimulation which ceases when pH_i falls below a value of about

6.3. However, even though a pH_i of 4.8 was predicted according to the Donnan equilibrium, the lactic acid production was nearly normal, indicating that the Donnan equilibrium does not control or greatly influence the H^+ distribution across the fibre membrane. As well, the experimental findings of Caldwell (1958) showed that the pH_i's expected from the Donnan relationship in response to depolarization do not occur.

In the past, two possible mechanisms have been proposed to account for the disagreement between measurements of pH_i and what is expected from the Donnan equilibrium. Fenn and Mauren (1935) originally cited the lack of homogeneity of the cell interior as a probable factor. The second proposal involved active extrusion of hydrogen ions which, in most cases, is the preferred explanation (Caldwell 1958).

3) Muscle Buffer Value

a) Background

In any consideration of acid base balance it

becomes immediately obvious that a well-defined intracellular buffer value is of prime importance. This value is of special significance when considering skeletal muscle which is such a large proportion of the whole body and thus, will contribute greatly to the overall buffering. The most common measure of the buffer value of a solution is that introduced by Van Slyke (1922) in which "β" equals the amount of free H^+ or OH^- to be added to a volume of a solution in order to produce a unit change in the pH value.

Due to its special properties, the CO_2-bicarbonate buffering system must be considered separately from what may be termed "non-carbonic buffers". However, some of the early studies were performed without taking this separation into consideration. For example, Furusawa and Kerridge (1927) added small amounts of acid or base to minced cat gastrocnemius, to obtain a buffer value of 52 mM acid or base per pH unit per kilogram tissue at a pH_i of 6.5. Unfortunately, the gaseous

CO_2 equilibration was not given, so that the exact non-carbonic buffering cannot be determined. Results comparable to this have been obtained by others using similar techniques (Bate Smith, 1938; Eckel et al 1959).

Other means of measuring the intracellular buffering of muscle involved the use of the DMO method of Waddell and Butler (1959) to measure pH_i. Some of these measurements were made on muscle in situ (Brown and Goott, 1963; Clancy and Brown, 1966; Schloerb et al, 1967; Burnell 1968). Other experiments were performed on the rat diaphragm equilibrated in vitro (Adler, Roy and Relman, 1965a and b; Heisler and Piiper, 1972). In these studies, the muscles were allowed to equilibrate with different levels of pH and $[HCO_3^-]$. Then the change in pH_i was determined. The results all produced an intracellular buffering of less than 20 meq/pH unit per litre muscle water.

As suggested by Heisler and Piiper (1971), there was a possibility in these types of studies for exchange of substances, particularly those important for buffering (e.g., HCO_3^-, H^+ or OH^-), from the intracellular space.

b) "Apparent" versus "True" Buffer Value

The work of Adler, Roy and Relman (1965a and b) indicated that the regulation of the acid base balance of muscle fibres is not simply a matter of intracellular chemical buffering. An additional contributing mechanism might be the transfer of HCO_3^- (H^+ or OH^-) after change in pCO_2. Clancy and Brown (1966) pointed out the consequences of such a movement of HCO_3^- between the cell interior and the extracellular space. Under these circumstances, they postulated, there will be an underestimation of the muscle buffer value, determined by measuring pH_i with DMO and calculating intracellular bicarbonate from the Henderson-Hasselbach equation. To test this hypothesis, Heisler and Piiper (1971) equilibrated rat diaphragm homogenates with various levels of pCO_2 and measured the change in pH. Assuming that all the buffering originates from inside the fibres and the destruction of tissue did not affect the buffer value, they calculated a buffer value for the intracellular space of the rat diaphragm to be 67 meq/pH unit per kg cell water. Using a similar

technique, Lai et al (1973 c) obtained a buffer value for rat thigh muscle of 50-60 meq/pH unit per kg muscle water.

Subsequently, evidence began to accumulate which supported the transfer of HCO_3^- as a reason for the discrepancy between the different measurements of muscle buffer value (Heisler and Piiper, 1972; Izutsu , 1972; Lai et al 1973 a, b and c; Heisler 1975; Beattice et al 1976). In 1972, Heisler and Piiper used the distinctive terms, "apparent buffer value" and "true chemical buffer value". For rat diaphragm, they calculated these values to be 20 meq/pH unit per kg cell water and 68 meq/pH unit per kg cell water. They found that, on the average, 71% of the bicarbonate formed or decomposed in response to the action of intracellular non-bicarbonate buffers was transferred to (or from) the extracellular space.

However, not all studies supported these findings. Using improved pH microelectrodes with recessed tips, Aickin and Thomas (1976, 1977) calculated a non-bicarbonate buffer value of 45 meq/pH unit per

litre cell water in the isolated soleus muscle of the mouse. They did not accept the higher value of Heisler and Piiper (1972), disagreeing with the latter's assumption that transfer of HCO_3^- occurred after exposure to different levels of CO_2.

Using the DMO method they developed for measuring pH_i, Waddell and Butler (1959) examined the effects of changes in extracellular pH by sampling thigh muscles of anaesthetized dogs. They concluded that the greatest changes in pH_i of muscle are those produced by raising CO_2 tension of blood and that metabolic acidosis was less effective in this process. Other studies have indicated that the intracellular pH and bicarbonate concentration of skeletal muscle do not change during metabolic acidosis in the intact animal (Wallace and Hastings, 1942; Wallace and Lowry, 1942; Brown et al 1967).

However, Adler, Roy and Relman (1965a and b) working with isolated rat diaphragm, were the first to use a complete range of bicarbonate and $p CO_2$ values in order to establish the relationship of extracellular pH to pH_i. Heisler (1975), also using incubated

rat diaphragm, performed similar experiments. The results from the two studies can be compared in fig. 1 (A and B).

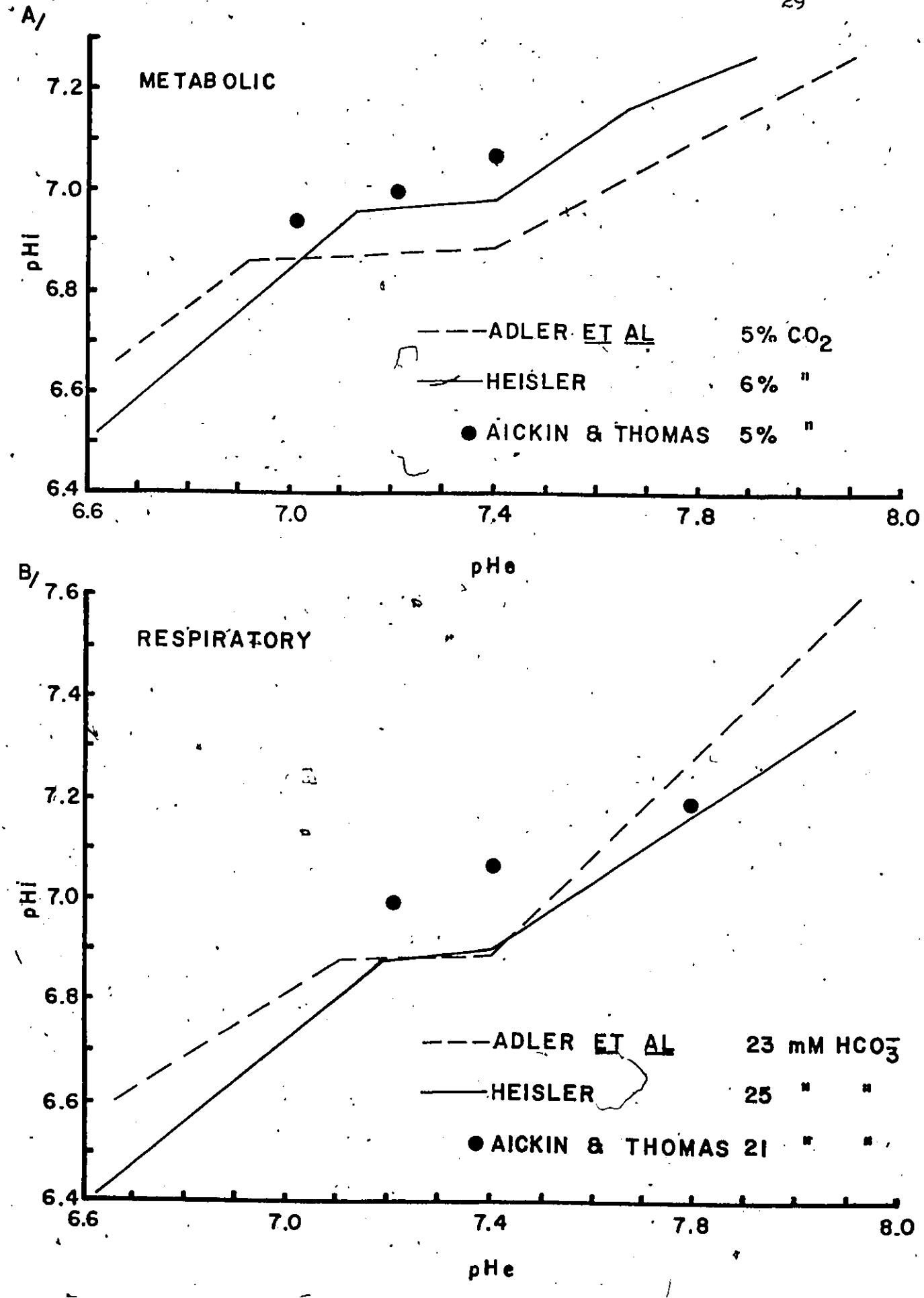
The top diagram illustrates the effects of metabolic acidosis and alkalosis on pH_i, maintaining pCO_2 constant and varying HCO_3^- to produce the pH_e range indicated. The lower graph, however, reflects the intracellular pH change in response to respiratory acidosis and alkalosis at a constant $[HCO_3^-]$. The results of Adler et al (1965 a) and Heisler (1975) are similar, through the pH range considered. All graphs show a limited plateau area moving in an acidic direction from the normal pH_e. During both metabolic and respiratory acidosis, Adler et al have a plateau region which extends slightly further than that of Heisler. At both ends of the plateau, the pH_i changes rapidly with pH_e at the rate indicated by the slopes of the lines.

The existence of this plateau region indicates that over a limited range of pH_i the flux of H^+, OH^+ or HCO_3^- differs from that predicted by the passive flux of HCO_3^-. Adler, Roy and Relman (1965) thought that the plateau could

Fig. 1.

A. Effect of metabolic acidosis or alkalosis on intracellular pH by various investigators. These results were obtained by using a constant pCO_2 and varying the concentration of bicarbonate in the bathing medium, The CO_2 level is indicated for each set of results. The results of Adler et al (1965) and Heisler (1975) are from rat diaphragm using the DMO method while Aickin and Thomas used microelectrodes on mouse soleus muscles.

B. Effect of respiratory changes in external pH on intracellular pH. In these experiments the pCO_2 was varied while bicarbonate concentration in the bathing solution was maintained constant. The constant $[HCO_3^-]$ is indicated for each set of results.


A/
METABOLIC
7.2
7.0
6.8
6.6
6.4
pHi
6.6
7.0
7.4
7.8
8.0
pHe
---ADLER ET AL 5% CO_2
—HEISLER 6% "
● AICKIN & THOMAS 5% "
B/
RESPIRATORY
7.6
7.4
7.2
7.0
6.8
6.6
6.4
pHi
6.6
7.0
7.4
7.8
8.0
pHe
---ADLER ET AL 23 mM HCO_3^-
—HEISLER 25 " "
● AICKIN & THOMAS 21 " "

be explained either by the existence of a limited barrier to the net flux of H^+, OH^+, or HCO_3^- ions or by compensatory changes in the internal metabolism of acids and bases. On the other hand, Heisler (1975) suggested that the simplest explanation for the essentially constant ranges of pH was to assume an active transport mechanism of ions involved in the acid-base equilibrium of cells. The active transmembrane transfer of HCO_3^- (H^+ or OH^-) has also been postulated as a mechanism of cardiac muscle buffering (Gonzalez and Clancy, 1975; Strome and Gonzalez, 1975).

Also included in fig. 1 (A and B) are the results of Aickin and Thomas (1977) from isolated mouse soleus measured with recessed tip pH microelectrodes. Their normal pH_i was slightly higher than those of the DMO technique. This was thought to be due to having fibres in better condition than most of the whole muscle preparations and avoiding the inclusion of the pH of cellular organelles. This was true also of Khuri

et al (1974, 1976) using HCO_3^- selective micro-electrodes, giving a pH_i of 7.03 on rat thigh muscles.

Aickin and Thomas did not measure a plateau region under slightly acidotic conditions. Their results showed that the change in pH_i is about ten times faster when the CO_2 level is changed rather than the bicarbonate concentration although the final pH_i is the same. They suggested that this indicated the role of CO_2 in rapidly moving H^+ ions across the fibre membrane and that the change in pH_i made by changing $[HCO_3^-]$ was due to a lack of movement of H^+ ions and/or HCO_3^- ions across the membrane. Aickin and Thomas (1977) suggested that the lack of a plateau region for the plot of pH_i with pH_e for their results compared with those of Adler, Roy and Relman (1965) and Heisler (1975) could be explained by the inclusion of deteriorating, or even dead, innermost fibres of the in vitro preparations in the determination of pH_i.

A third type of measurement has been made using DMO on in vivo muscle preparations. The majority of these studies have obtained results in agreement with the microelectrode observations. There was no observed plateau region for pH_i over a pH_e range of 7.0 to 7.4 (Schloerb, et al, 1967; Roos 1971; Poole-Wilson and Cameron, 1975; Gonzalez et al, 1976). The explanation for this discrepancy was based on a CO_2 gradient in the isolated non-perfused rat diaphragm preparation. If, due to thick tissues, the diffusion of pCO_2 is impaired, then this is a valid criticism of in vitro preparations.

From these types of studies the crucial question remains concerning the exchange of substances important to buffering (e.g., HCO_3^-, OH^- and H^+) in response to changes in the levels of extracellular pCO_2 and $[HCO_3^-]$. This question has been looked at in another manner; that of measuring the permeability of the ions, in particular HCO_3^-, and their possible effects on membrane potential.

4) Permeability of HCO_3^- Ions

Mainwood (1968) measured transmembrane potentials in frog sartorius muscles that had been washed in a buffered sucrose solution. When CO_2 was increased the transmembrane potential was reduced. It was suggested this may have been due to an efflux of bicarbonate ions. Other experiments similar to this were performed by Woodbury (1971) also using frog sartorius in which Cl^- had been replaced by an impermeant ion. His measurements of transmembrane potential and resistance indicated that the permeability of HCO_3^- is appreciable. Woodbury approximated the ratio of bicarbonate permeability to chloride permeability to be about 0.1.

Hutter and Warner (1967a and b) demonstrated the pH dependence of chloride conductance. Woodbury and Miles (1973) stated that HCO_3^- acted as a Cl^- like anion which also exhibited a conductance which was sensitive to pH.

A review of the literature raises an im-

portant question: to what extent and in what form is transmembrane movement of acids and bases occurring in muscle fibres in response to changes in muscle tissue as a result of alterations in CO_2 level and bicarbonate concentration?

In general, what appears to be missing from the research relating to acid-base balance for mammalian muscles are studies for muscle tissue in vitro in which the addition or removal of acid or base from the external bathing solution is measured. From such studies it would also be possible to determine a permeability coefficient for HCO_3^- based on changes in HCO_3^- flux (having made the assumption that the acid base exchange is due to transmembrane HCO_3^- movements) in response to changes in the CO_2 level and $[HCO_3^-]$.

Another important aspect which direct measurement of transmembrane fluxes could provide information about is the effect of the transfer of metabolic acids such as lactate and pyruvate on acid-base exchange. One of the specific problem

areas revealed in the literature is the extent to which acid-base exchange is affected by trans-membrane movement of the lactate ion compared with the effect of transfer of the lactic acid molecule.

C. Aims and Objectives

1. To develop a method for the sensitive measurement of lactate efflux and acid base exchange in the isolated rat diaphragm.

2. To make careful observations during the control state of the muscle and then during the changes induced by respiratory and metabolic pH changes in the bathing medium.

3. Relate the characteristics of lactate efflux and acid base exchange to the theoretical behaviour predicted by the results of others.

4. To monitor the contractile state to ensure

a viable preparation and observe any effects of acid base transfer on tension.

5. To develop a theoretical model for acid base exchange.

CHAPTER II

Methods and Materials

A. Muscle Preparation

1) Suitability of the Rat Diaphragm

The muscle preparation is of critical importance. A tissue was needed which was of sufficient mass to obtain reliable measurements yet thin enough to allow diffusion in the shortest possible time. It should also be representative of skeletal muscle. Another consideration is that it must be able to survive for long periods of time when removed from the animal. It is also necessary to be able to attach the muscle so that it can be exposed to sufficient oxygen and metabolic substrate supply as well as obtaining isometric tension measurements as a measure of the relative contractility. The rat diaphragm was considered to be the most advantageous in these circumstances.

The rat diaphragm preparation was first used by Bülbring (1946) since it appeared to be

thin enough to allow oxygenation. In her preparation, a strip of diaphragm was suspended in an organ bath with an intact phrenic nerve supply for indirect stimulation of the muscle. The rat diaphragm has also been used for many studies of carbohydrate metabolism in incubation flasks without any stimulation (e.g., Walaas and Walaas, 1950; Haugaard et al, 1951). A continuous perfused preparation was also developed by Brownlee and Straughan (1957). Therefore, the rat diaphragm has been used extensively for many different types of studies which have proven its usefulness as an experimental preparation.

Creese et al (1958) using 200-280 gram rats, were able to demonstrate by recording membrane potentials that it is possible to oxygenate the isolated diaphragm in vitro. Neptune et al (1960) demonstrated the necessity of glucose in the medium to maintain contraction of the rat diaphragm.

That the rat diaphragm might be unique

and not representative of skeletal muscle has been suggested by several studies (Beatty et al, 1960; Pannier et al, 1970; Heisler, 1975). Its actual fibre composition is that of a mixed muscle with many large, "white" fibres with a low mitochondrial content (Gauthier and Padykula, 1966). However, its speed of contraction is actually slower than the mouse diaphragm which consists entirely of small "red" fibres with abundant mitochondria. This is thought to be a functional adaptation produced by the different rates of breathing of the two animals (Bass, Gutmann and Hanikova, 1971).

2) Method of Preparation

There were several different designs and approaches in the development of an efficient muscle bath and sensitive pH measuring system. The description of the methods will for the most part deal with the final version of the system with reference to the improvements to be included in each section.

In all experiments male Sprague-Dawley rats, 150-200 g were used. The rats were killed by a blow to the back of the head. The chest cavity was exposed by a transverse cut across the ribs approximately 3 mm anterior to the diaphragm. In all later experiments a 30 ml glass syringe with polyethylene tubing on a #18 needle was used to wash Tyrode solution into the descending aorta (fig. 2). By filling and emptying the syringe 3 times, a total of 90 ml was forced through the circulatory system of the rat. In earlier experiments the diaphragms did not undergo this rinse with Tyrode. It was thought that the residual blood in the diaphragm might be affecting the results and that the rinse would remove this blood. However, there was no change in the results with or without the rinse although this procedure was continued for all the subsequent experiments.

After this rinse the whole diaphragm with rib attachments was removed and placed in a

Fig. 2.

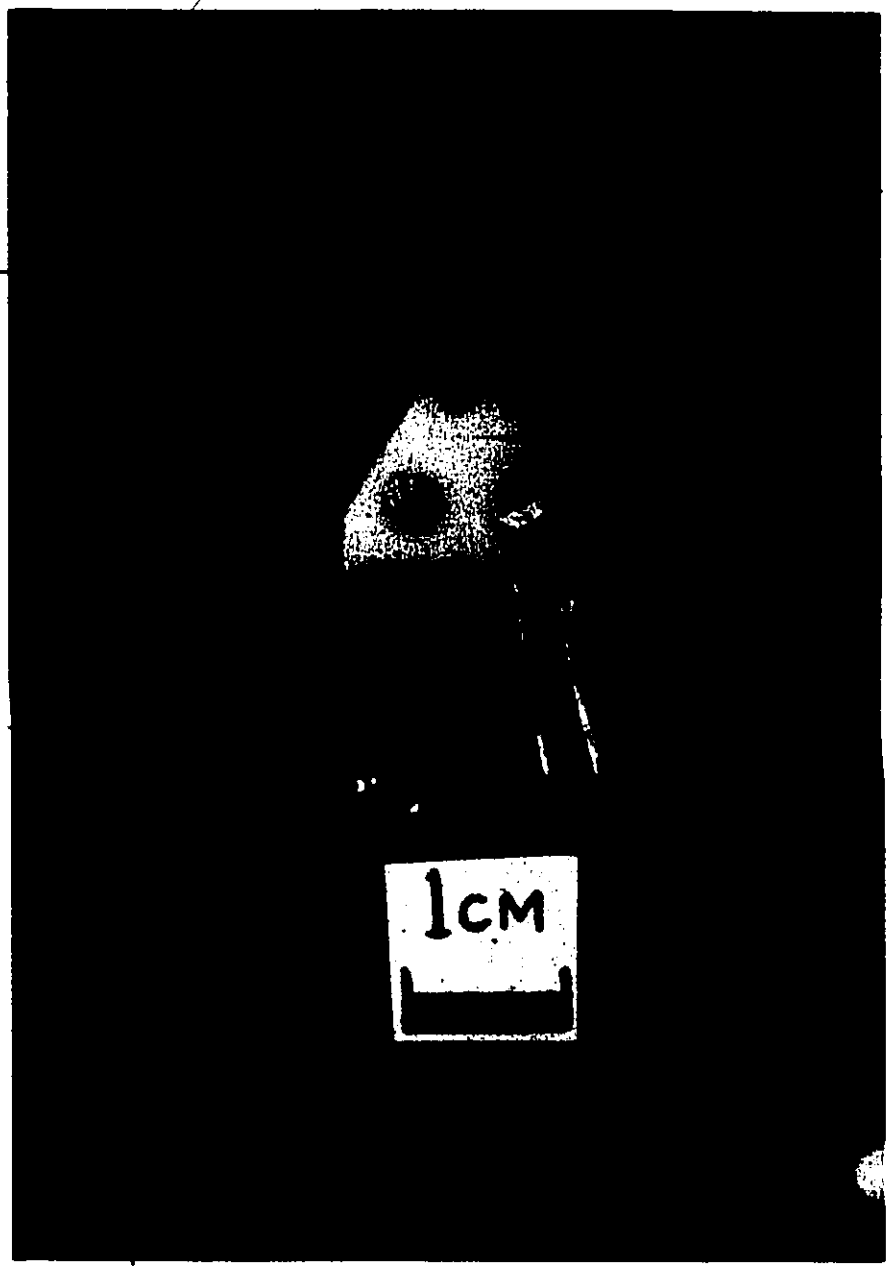
Photograph of the dissection of the diaphragm before removal from the rat. A section of PE tubing is inserted in the aorta. A 30 ml glass syringe (not shown) is fitted to the other end of the tubing.

wax dissecting dish filled with Tyrode. A strip of diaphragm about 15 mm wide was cut from the left side of the diaphragm close to the midline. The portions of ribs still attached to this strip of muscle were carefully removed by running the edge of a razor blade along the surface of the bone. Earlier experiments did not have all the pieces of ribs removed but were used to attach the muscle to a rigid support for the measurement of tension. However, the question was raised that buffers (e.g. phosphate) might be washed from the bone which would affect the net acid efflux results. After careful removal of the bone initial tensions were obtained which were comparable to those in which the ribs were used to fasten the muscle.

Using a small surgical needle and nylon thread the rib edge of the diaphragm strip was tied in 5 or 6 places to a small stainless steel bar 16 mm long. The central tendon was fastened with a small plexiglass clamp (fig. 3).

Fig. 3.

Photograph of the diaphragm strip with clamp and bar attachments.


1cm

This whole arrangement was placed in the muscle bath (fig. 4). The metal bar was fitted into small grooves for rigid support while the plexiglass clamp was connected by a thin metal tube to a light chain suspended from the tension transducer.

B. Preparation of Solutions

All solutions were prepared using deionized water and Fisher Certified Reagents. They were made fresh on the day of the experiment from 1 M stock solutions (Na Cl stock solution was 4 M). The glucose was weighed separately each time. The composition of the solutions is shown in Table I.

The solutions were equilibrated with appropriate mixtures of CO_2 and O_2 gas to obtain the desired pH except for the anoxic experiments in which N_2 replaced the oxygen.

Fig. 4.

Photograph of the diaphragm strip in place in muscle bath.

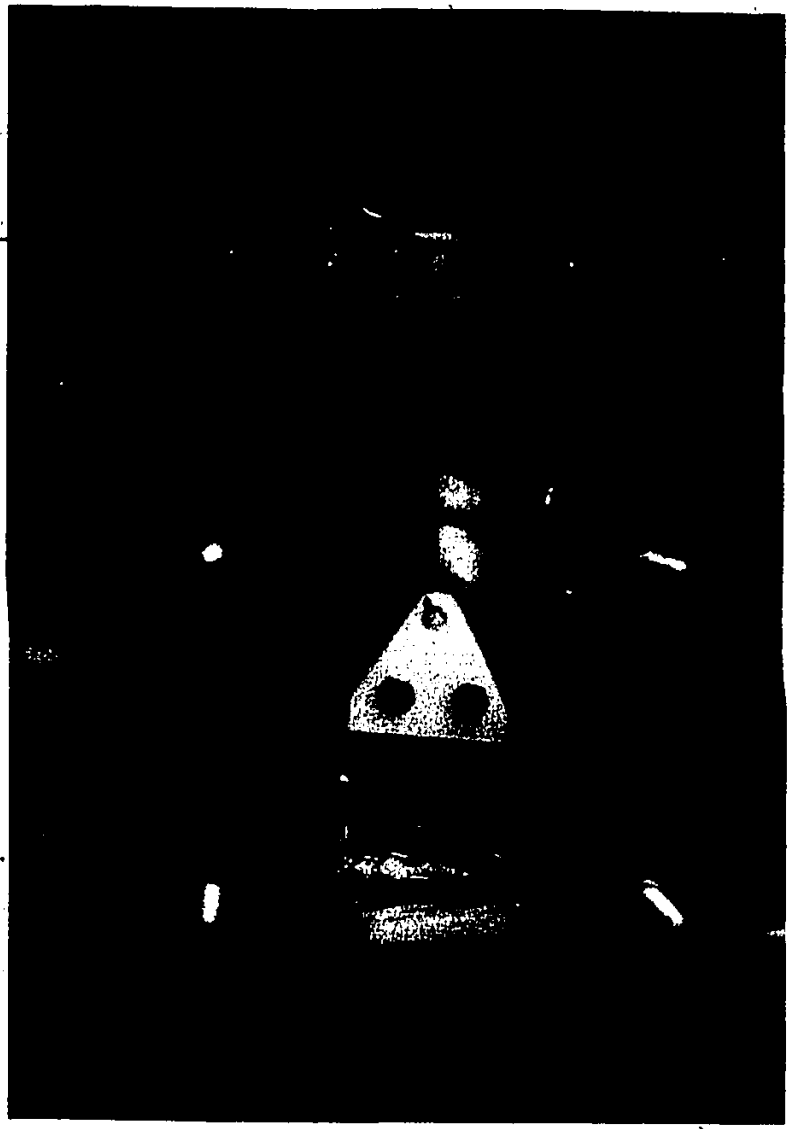


TABLE I

COMPOSITION OF SOLUTIONS (mM)

Solution	Normal	low HCO_3^-	high CO_2	median CO_2	low CO_2	high CO_2 low HCO_3^-
KH_2PO_4	1.2	1.2	1.2	1.2	1.2	1.2
$CaCl_2$	2.5	2.5	2.5	2.5	2.5	2.5
$MgSO_4$	3.1	3.1	3.1	3.1	3.1	3.1
KCl	3.5	3.5	3.5	3.5	3.5	3.5
$NaHCO_3$	28	6.6	28	28	28	6.6
NaCl	117	138	117	117	117	138
Glucose	5.5	5.5	5.5	5.5	5.5	5.5
CO_2	5%	5%	20%	10%	2%	20%
pH	7.47	6.85	6.88	7.19	7.89	6.24

C. Apparatus and Design of Experiments

1) Description of Apparatus

A diagrammatic representation of the apparatus associated with the muscle bath is shown in fig. 5 (A and B). The muscle bath chamber was milled out of 1/2" plexiglass. The back and side walls were made very thin so that the large water bath behind could equilibrate solutions in the muscle bath to within 1°C of the temperature in the warming bath. The time for equilibration was less than 2 minutes. The temperature in the muscle bath was maintained at 36°C for all experiments.

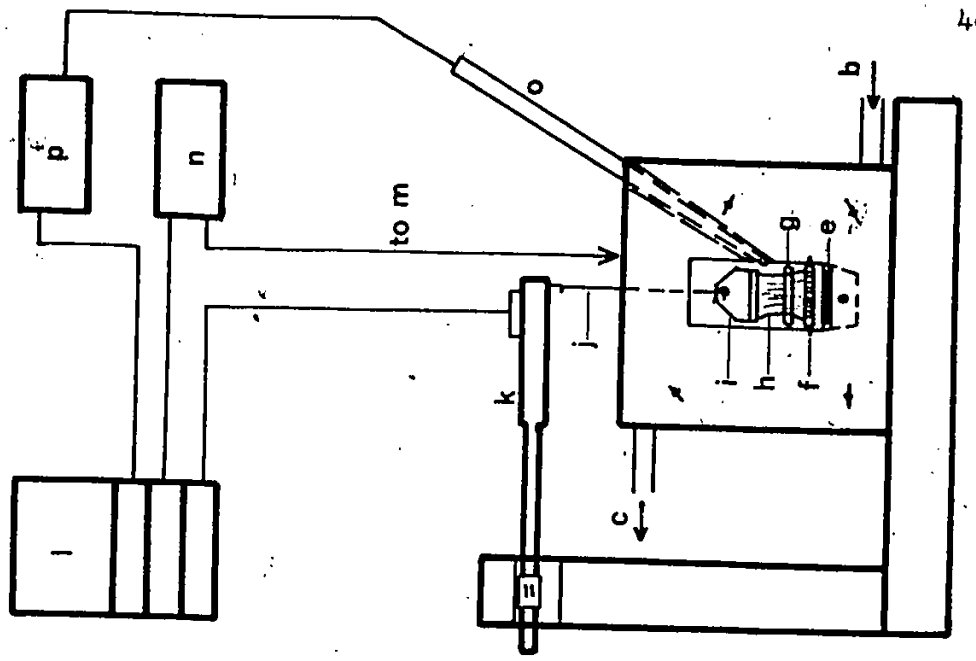
The various gas mixtures were pre-equilibrated for temperature and water content in systems similar to that which is described for the pH test bath. The gas then passed through a steel tube which coils throughout the large warming bath before entering a small chamber below the muscle bath. A strip of scintered glass separated this smaller chamber from the

Fig. 5.

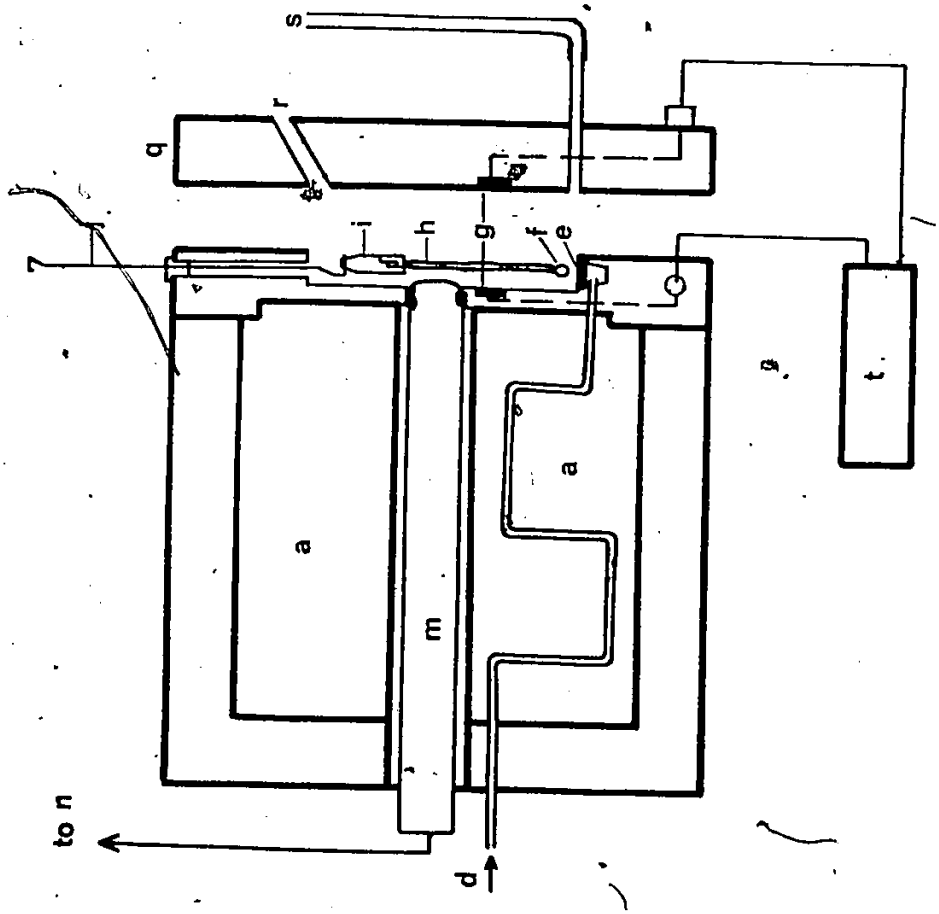
A) Crossection of a side view of the muscle bath and frontspiece, illustrating pO_2 electrode position, gas bubbling system, muscle clamp, and stimulating electrodes arrangement.

B) Frontal view of the muscle bath illustrating position of the pH electrode and the stimulating and tension recording apparatus.

a	warming bath	k	tension transducer
b	warm water inlet	l	polygraph
c	warm water outlet	m	pO_2 electrode
d	gas inlet	n	pO_2 monitor
e	scintered glass	o	pH electrode
f	stainless steel bar	p	pH meter
g	platinum electrodes	q	frontspiece
h	diaphragm strip	r	bathing sol'n inlet
i	muscle clamp	s	bathing sol'n outlet
j	thin steel tubing	t	stimulator



B/



A/

muscle bath proper which allowed the gas to bubble evenly along the entire width of the muscle strip. In this manner a constant and even washing of the outer surface of the tissues was achieved as well as providing a quick and efficient equilibration of the bathing fluid with the various gas mixtures.

The frontspiece of the muscle bath consisted of a single 1/2" sheet of plexiglass. Solutions were injected with a 1 ml glass syringe through a hole in the front plate near the top of the muscle chamber. A 3 ml plastic syringe was used to withdraw solutions from polyethylene tubing attached to a steel tube which penetrated the frontspiece at a point coinciding with the lower corner of the muscle bath.

Tension was recorded on a Grass Model 7 Polygraph employing a Grass Force-Displacement Transducer Model FT 03C. The maximum working range of this transducer was

0.01 to 200 grams when used with the appropriate spring. A Tektronix Model 121 N Oscilloscope connected to the Driver Amplifier output of the polygraph was used to monitor the shape of the individual tetanic contractions.

The muscles were stimulated with a Grass Model S4 Stimulator connected to platinum electrodes inserted on each face of the muscle bath. The electrodes were 3 mm wide and extended the entire width of the inside of the muscle bath at a point halfway down the length of the muscle. To control the frequency and the duration of the tetani a Tektronix Type 160 A Power Supply was used to drive a modified Type 162 Waveform and Type 161 Pulse Generators which supplied the external control to the Grass Stimulator.

Smooth tetani with a flat plateau were obtained with 0.5 msec pulses at a frequency of 200/sec for a total duration of 0.2 sec. Maximum isometric tensions were achieved at the start of each experiment by gradually

increasing the stimulus voltage and then resting tension of the muscle until there was no further increase in contraction height. The stimulus voltage was then increased by another 30% to assure maximum isometric contractions throughout the experiment.

Recording electrodes for measurement of percent oxygen saturation and pH were also fitted into the muscle bath. The percent oxygen saturation was measured with a Y5l Model 53 Oxygen Monitor. A permanent record was also obtained on the Grass polygraph.

The pH was monitored with a Microelectrodes Micro-Combination Glass pH Probe connected to an Orion Model 80l Digital pH Meter. The pH meter was also connected to a separate channel on the polygraph for a permanent record of pH in the muscle bath.

Initially this recording system was used to determine the small changes in pH due to net acid efflux from the muscle. However, the pH

was measured in a remote test bath for the following five reasons: a) the ΔpH is essentially a comparison between the pH of the Tyrode before and after muscle equilibration which is only done accurately if samples are alternated; it is not possible to do this directly in the muscle bath, b) noise induced by the stimulus and polarizing currents and movements of the muscle upsets pH electrode measurements, c) small local changes in pCO_2 may arise due to muscle metabolism which would greatly influence ΔpH measurements even though the absolute pH level is very close to the predicted value, d) the small size of the chamber and the muscle shape could lead to small pockets which are not as well mixed as other parts; this would also seriously affect ΔpH, e) a test bath designed specifically for the measurement of pH permitted a finer control of temperature, gas bubbling, as well as the use of a larger, more stable pH electrode. These improve-

ments contributed to a more accurate and stable system for monitoring pH.

The pH electrode remaining in the muscle bath was used to measure gross changes in pH after a change in pCO_2 and bicarbonate concentrations.

2) Measurement of pH

The apparatus employed for a sensitive measurement of pH is shown in fig. 6. The chamber consisted of a tall, double-walled glass bath with a scintered glass bottom through which a gas mixture could be bubbled. This test bath was designed to conform to the measurements of the Radiometer GKS7304l Semi-Micro Combination pH Electrode.

The electrode was connected to a Radiometer Ph M 64 Research pH Meter which in turn was used to drive a Sargent-Welch Model TRG Recorder. The recorder was calibrated so that every 0.040 pH units resulted in a fullscale deflection.

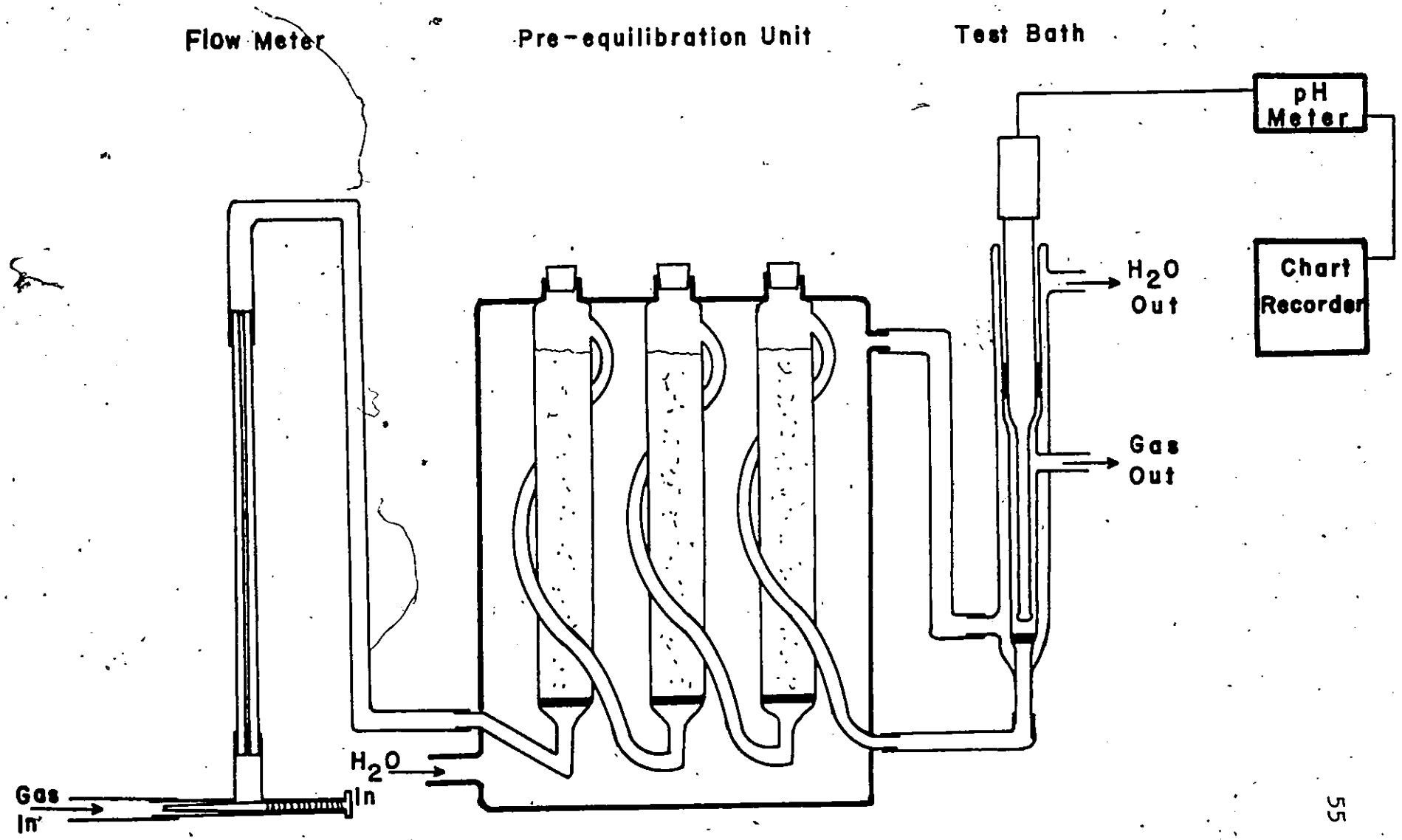
A fine control of temperature was necessary since a deviation of 0.1°C in the test bath resulted in a concurrent 0.001 pH deflection due to the change in CO_2 solubility. Therefore, a precise temperature was maintained with the use of a Heto Constant Temperature Circulator Model TGUL with an accuracy of ±0.05 to ±0.005°C.

However, it was also necessary to warm the gas mixture (95% O_2 and 5% CO_2) to the same temperature as the solution in the test bath. It was also important that this gas did not remove or contribute water to the Tyrode solution through which it was bubbled since a change in volume would alter the buffer concentration. To maintain temperature and water content of the gas mixture a "pre-equilibration unit" was designed.

This unit consisted of a large glass jar which enclosed three separate chambers as

Fig. 6.

Illustration of the design of the "pre-equilibration unit" used to warm and maintain water vapour of the gas to be bubbled through the test bath. The same apparatus was used to pre-equilibrate gases for the large reservoir baths and the muscle bath.


Flow Meter
Pre-equilibration Unit
Test Bath
pH Meter
Chart Recorder
H_2O Out
Gas Out
H_2O In
Gas In

illustrated in fig. 6. The large outer chamber contained water which was circulated through the water heater. The three smaller inner chambers were connected in series with glass tubing. The bottom of each small chamber was made of scintered glass and each was filled with isotonic NaCl. The gas came directly from its cylinder, flowed into the glass tubing and bubbled through the NaCl solution in each chamber consecutively before entering the test bath. A gas flow meter was used to set the rate of gas bubbling at 6 ml/minute.

Tyrode samples for both the muscle bath and the controls for the test bath were removed from large 150 ml double-walled reservoir baths which were also pre-equilibrated for both temperature and the appropriate gas mixture. In most of the experiments the Tyrode solution remained in the muscle bath for 10 minute periods. At the end of the 10 minutes

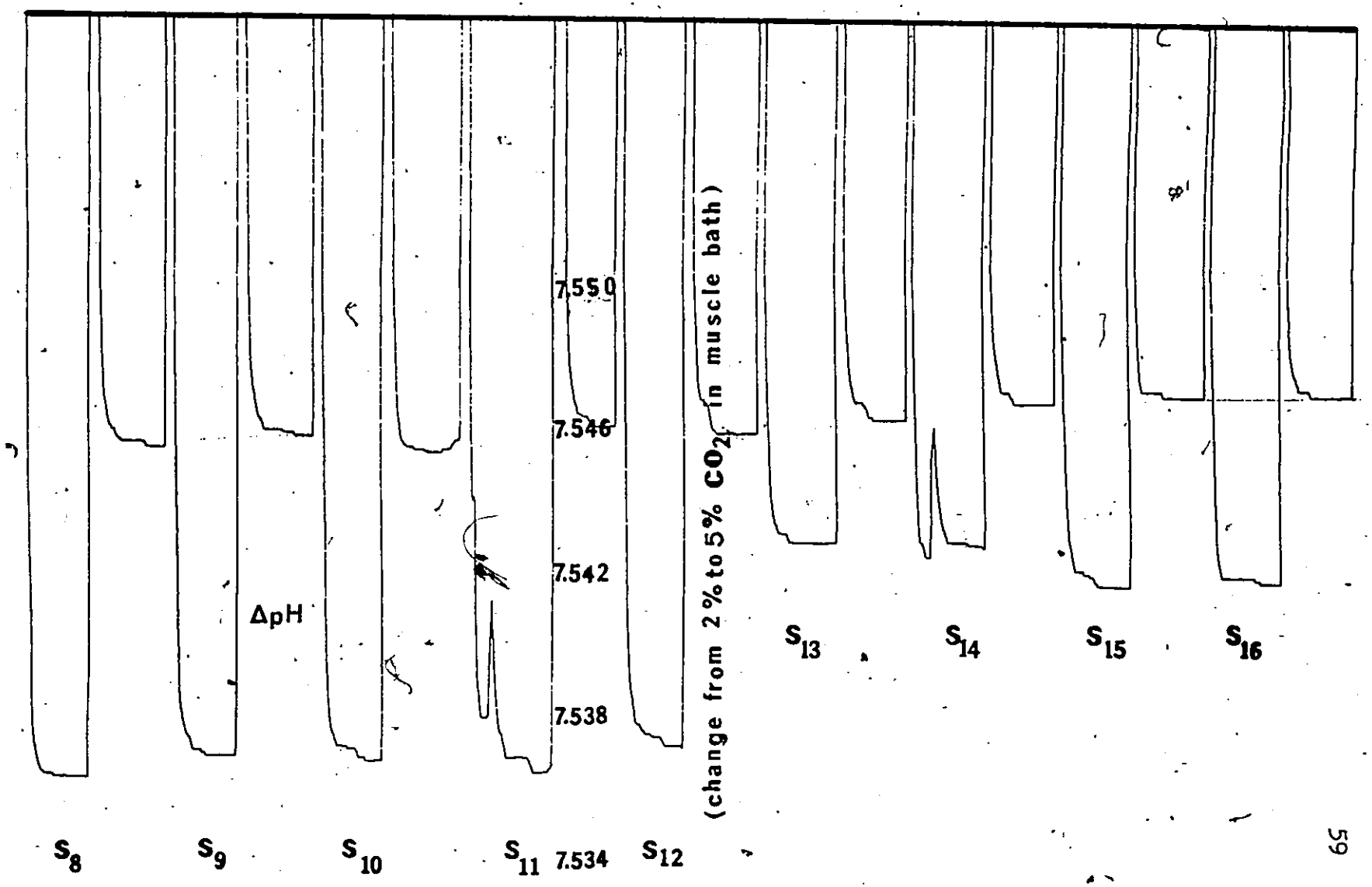
this solution would be removed and placed in the test bath. Fresh solution from the large reservoir baths would then be injected into the muscle bath.

The Tyrode sample taken from the muscle bath remained in the test bath for 5 minutes to obtain an accurate reading of the pH value. The sample was then removed and temporarily stored in a test tube on ice to be used for the assay of lactate and pyruvate. A control sample (Tyrode solution directly from the reservoir bath) was placed in the test bath and the pH recorded for 5 minutes. By that time the next 10 minute test sample was ready to be removed from the muscle bath and recorded. Therefore, in alternating 5 minute periods a control pH would occur before and after each test sample. The pH was taken as the difference between the test sample and the average of the two juxtapositioned control samples.

Fig. 7 shows the actual pH recording of a section of one experiment at a point where the muscle bath was changed from 2% CO_2 (pH 7.88) to 5% CO_2 (pH 7.47). As mentioned earlier the test bath was equilibrated continuously with a 95 and 5 percent mixture of O_2 and CO_2 respectively. Therefore the differences in pH are the result of the time the test solution spent in the muscle bath. At the beginning and end of this experiment (as in all experiments) a series of blanks was recorded during which time there was no muscle in the muscle bath. These blanks resulted in no significant deviation from the baseline given by the control samples. Thus, the pH indicated in fig. 7 is due to the presence of the muscle during each 10 minute test period. In order to determine from this change in pH the amount of acid added or removed from the solution it was necessary to develop an equation based on the two buffer

Fig. 7.

Section of the pH recordings from the test bath during a CO_2 change experiment. At the end of sample S_{12} the CO_2 in the muscle bath was changed from 2% to 5%. The CO_2 content of the gas bubbling through the test bath was not changed. The controls recorded between samples S_8 and S_{17} were taken directly from the same reservoir bath that the samples for the muscle bath were removed from. The time for each recording was 5 minutes.


7.550
7.546
7.542
7.538
7.534
ΔpH
(change from 2% to 5% CO_2 in muscle bath)
S_8
S_9
S_{10}
S_{11}
S_{12}
S_{13}
S_{14}
S_{15}
S_{16}

systems (bicarbonate and phosphate) used in the bathing fluid. The development of this equation is given in the appendix.

3) Experiments Involving Change in pCO_2

The CO_2 change experiments had a duration of 3 hours. In the first hour the muscle was equilibrated with a normal gas mixture (95% O_2 and 5% CO_2). At the beginning of the second hour the gas mixture was changed to one of the other three CO_2 concentrations (20%, 10% or 2%). A normal Tyrode solution was returned to at the beginning of the third hour. Throughout the experiment the Tyrode sample in the muscle bath was changed every 10 minutes resulting in 6 readings for every 1 hour period.

The change in gas mixtures was facilitated by using a second gas equilibration unit and reservoir bath pre-equilibrated with the new pCO_2.

4) Experiments Involving Change in $[HCO_3^-]$

The procedure for changing the bicarbonate concentration is complicated by the necessity of removing completely all traces of the previous solution. It was necessary to compromise between taking time to wash out the previous solution containing a different bicarbonate concentration and losing the results during that time of the initial changes in the muscle efflux. A marker system was used to compensate for the effect that an incomplete wash would have on the pH results.

In two experiments a double label of ^{14}C sucrose and ^{3}H inulin was used. In later experiments only ^{14}C sucrose was employed. All radioactive material was obtained from New England Nuclear.

The normal solution in one of the reservoir baths was labelled with high levels

of approximately 2 x 10^6 DPM's/ml of ^{14}C sucrose and 1.5 x 10^6 DPM's/ml of ^{3}H inulin. The six samples in the first hour contained radioactive label. When the samples were removed from the muscle bath approximately 150 ul was placed in a small test tube on ice while the remainder was put in the test bath to obtain a pH reading. From the 150 ul aliquot, two 50 ul samples were taken and placed in separate scintillation vials.

The Tyrode with the different bicarbonate concentration contained no label. The muscle bath was given a quick 1 ml rinse with the new Tyrode immediately after the last sample from the first hour was removed. This removed approximately 80-90% of the label. The rinse was followed by a 50 ml washout of the muscle chamber with pre-equilibrated solution from the reservoir bath containing the new $[HCO_3^-]$. This washout had a duration of 5 minutes and resulted in the removal of

approximately 99% of the previous solution. Thereafter, sampling took place as usual for the next 60 minutes. Two 50 ul samples for counting were removed from the 1 ml rinse solution, the washout solution, and each of the six samples from the second hour. Fig. 8 is an example of the results of a typical washout curve for a ^{14}C-sucrose label in an experiment with a $[HCO_3^-]$ change.

The procedure was the same when returning to normal Tyrode from low bicarbonate, although it was not necessary to remove samples for counting the label in the third hour. However, in one experiment the low bicarbonate Tyrode was labelled and sampling for counting took place during the second and third one-hour periods.

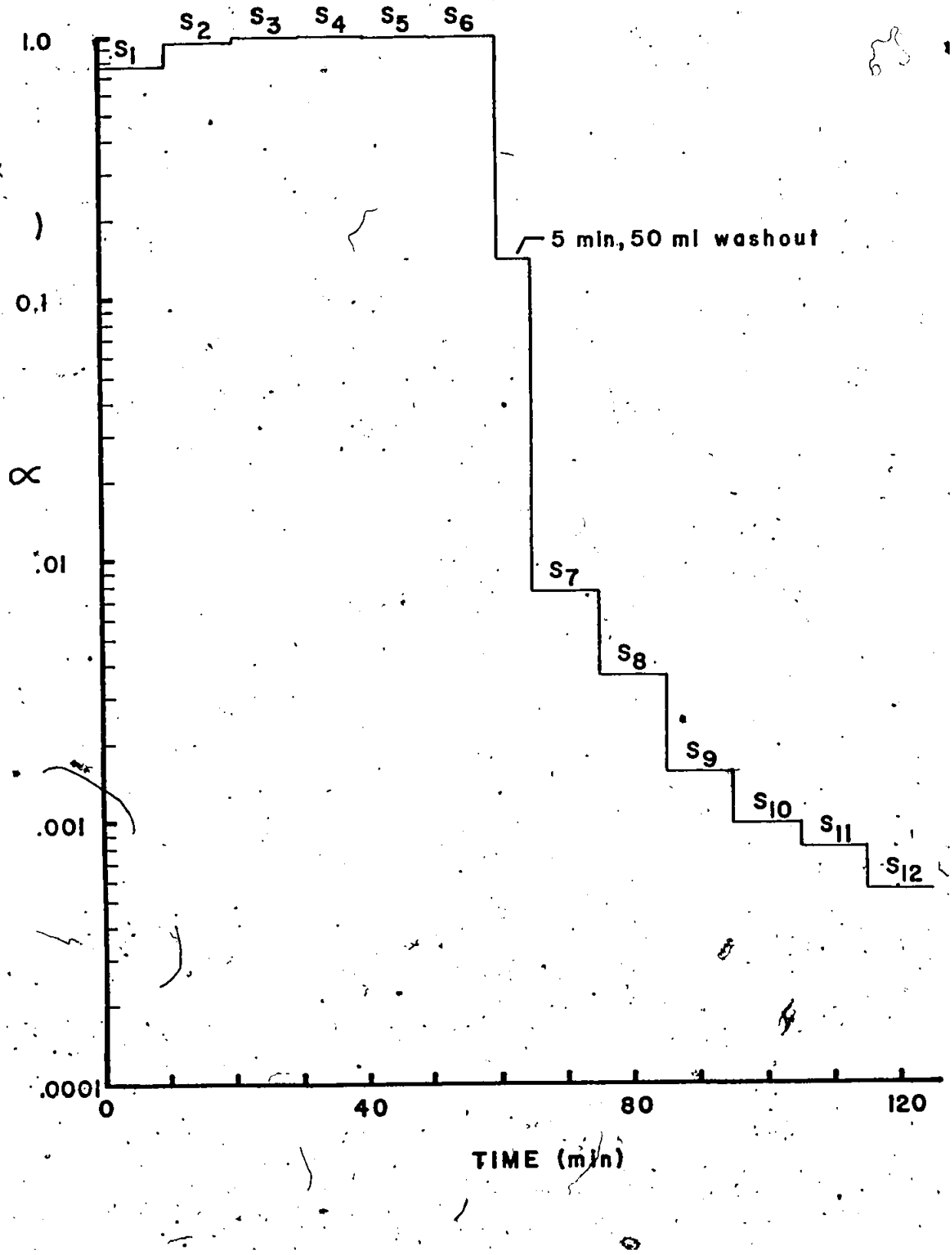
Ten mls of scintillation cocktail (New England Nuclear, Formula 950A) and 1 ml deionized water were added to the 50 ul

Fig. 8.

Illustrates the washout curve for a ^{14}C-sucrose label during a $[HCO_3^-]$ change experiment.

$$\alpha = \frac{\text{CPM in sample from muscle bath}}{\text{CPM in labelled reference solution}}$$

is plotted against time. The CPM in the labelled reference solution was approximately 2×10^6/ml.


1.0
0.1
.01
.001
.0001
∝
S_1
S_2
S_3
S_4
S_5
S_6
5 min, 50 ml washout
S_7
S_8
S_9
S_{10}
S_{11}
S_{12}
0
40
80
120
TIME (min)

samples in the scintillation vials. The samples were counted on a Nuclear-Chicago, Mark II Liquid Scintillation System. CPM's were determined using a Wang Computer, Model 600. The calculations necessary to convert the ^{14}C-sucrose counts correcting acid base exchange are given in the Appendix.

Corrections for unexchanged fluid should also be carried out for the experiments in which CO_2 is changed. This is true for both the lactate and change in H^+ ion concentration measurements. However, this was considered unnecessary. The concentration of these substances is small, therefore the 90% exchange obtained in a single rinse was sufficient to reduce residual amounts to an insignificant level. For example, if lactate efflux from a muscle in normal Tyrode is resulting in a bath concentration of 100 nM/ml at the end of a 10 minute period, then removing 90% of a 1 ml sample results in about 10 nM of lactate remaining. When another ml of fresh

Tyrode solution is added to the bath the initial concentration of 10 nM/ml is below the lowest level at which lactate can be significantly measured. A similar argument was used to justify the lack of correcting for residual amounts of H^+ ions at a different concentration in the muscle bath solution before the change to a new PO_2 level.

D. Chemical Assays

Lactate and pyruvate measurements were determined enzymatically by making use of the ability of lactate dehydrogenase (LDH) to interconvert these two substances. The lactate reaction adapted from the methods of Olson (1961) and Bergmeyer (1965) took place in a semicarbazide hydrochloride buffer at pH 10.0. Absorbence was measured spectrophotometrically at 340 mμ due to the change in the coenzyme NAD to NADH. Using the reverse reaction pyruvate was converted to lactate in a triethanolamine HCl buffer at pH 7:5

(Bergmeyer, 1965; Rosenberg and Rush, 1966).

Enzymes and coenzymes for these assays were obtained from Boehringer Mannheim.

To determine if the buffering capacity of the solution was being changed by the addition of phosphates from the muscle, a sensitive measurement of phosphate content was needed. A Fiske-Subbarow reagent was used and spectrophotometric measurements were made against phosphate standards at 660 mμ in 1 ml microcuvettes.

All spectrophotometric readings were taken on a Unicam Spectrophotometer model 800.

CHAPTER III

Results

The observations of steady state output of lactate and H^+ will be presented first in the results. Following this, the results of a single experiment will be considered to illustrate what measurements were made and how graphs and relationships were derived from them. The rest of the results will be grouped according to the type of experiment. At the end of this section there will be included some miscellaneous experiments and measurements. These were performed in order to support the reliability of the apparatus and method developed for the project and also to relate the results obtained to those of others.

A. Steady State Lactate and H^+ Output

Immediately after being placed in the muscle bath all diaphragm strips underwent a

150 ml washout with normal Tyrode at pH 7.47. They were then allowed to equilibrate for at least 30 minutes before the 10 minute changes began and readings were taken. The washout and 30 minute equilibration were necessary to allow initially high lactate efflux levels to decline to a constant value. As indicated in the discussion this is a characteristic typical of rat diaphragm preparations.

Following the 30 minute equilibration, all muscles then had a one hour period in normal Tyrode with the usual stimulation rate of 1 tetanus every 200 seconds to establish a baseline for the various parameters to be measured. The first 60 minutes of each experiment were considered to be the control conditions for this muscle preparation. During this period, there is a steady net acid efflux of about 250 nM/g per minute. Lactate efflux was approximately 130 nM/g per minute while pyruvate was about 10% that of the lactate

efflux during this time. During this period the efflux of lactate and pyruvate is about 60% that of the net acid efflux.

The possibility that buffers such as phosphate diffusing from the muscle may lower the pH was considered. Two tests were carried out which have eliminated this possibility. The first was a direct measurement of phosphate. Initially, all Tyrode solutions contained 1.2 mM KH_2PO_4. After equilibration with a muscle for 10 minutes all phosphate measurements of extracellular fluid were less than 1.3 mM. The value of 1.3 mM was compared with 1.2 mM phosphate in theoretical calculations of H^+ from a broad range of ΔpH values. The results showed less than 1% variation in calculated H^+ values for both 28 mM HCO_3^- and 6.6 mM HCO_3^- Tyrode solutions.

Secondly, titration curves were made, comparing original Tyrode solution with extracellular fluid collected over a 30 minute period. The pH was reduced to 5.0 with HCl and the

solution titrated back to 7.5 in a CO_2 free atmosphere. Fig. 9 illustrates the results of these titrations. It can be seen that there is a difference of less than 20 nM NaOH required for the muscle effluent and the original solution to reach any particular pH. This excludes the possibility that any buffer from the muscle or weak acid with a $pK > 5$ contributes significantly to the acid shift.

Muscle tension generally showed an initial fall but invariably levelled off to a decrease of about 1% every 10 minutes. This was taken as an indication of a suitable supply of substrate (glucose) for metabolic demands, the absence of major fibre damage, and the availability of suitable oxygenation.

B. Results of a Single Experiment

The results of a single experiment are illustrated by the graphs in fig. 10. In this

Fig. 9.

Titration curves for comparison of muscle effluent (—x—), with original Tyrode solution (—•—). Extracellular fluid was collected over a 30 minute period, the pH reduced to 5.0 with HCl, then the solution was titrated back to pH 7.5 with NaOH in a CO_2 free atmosphere.

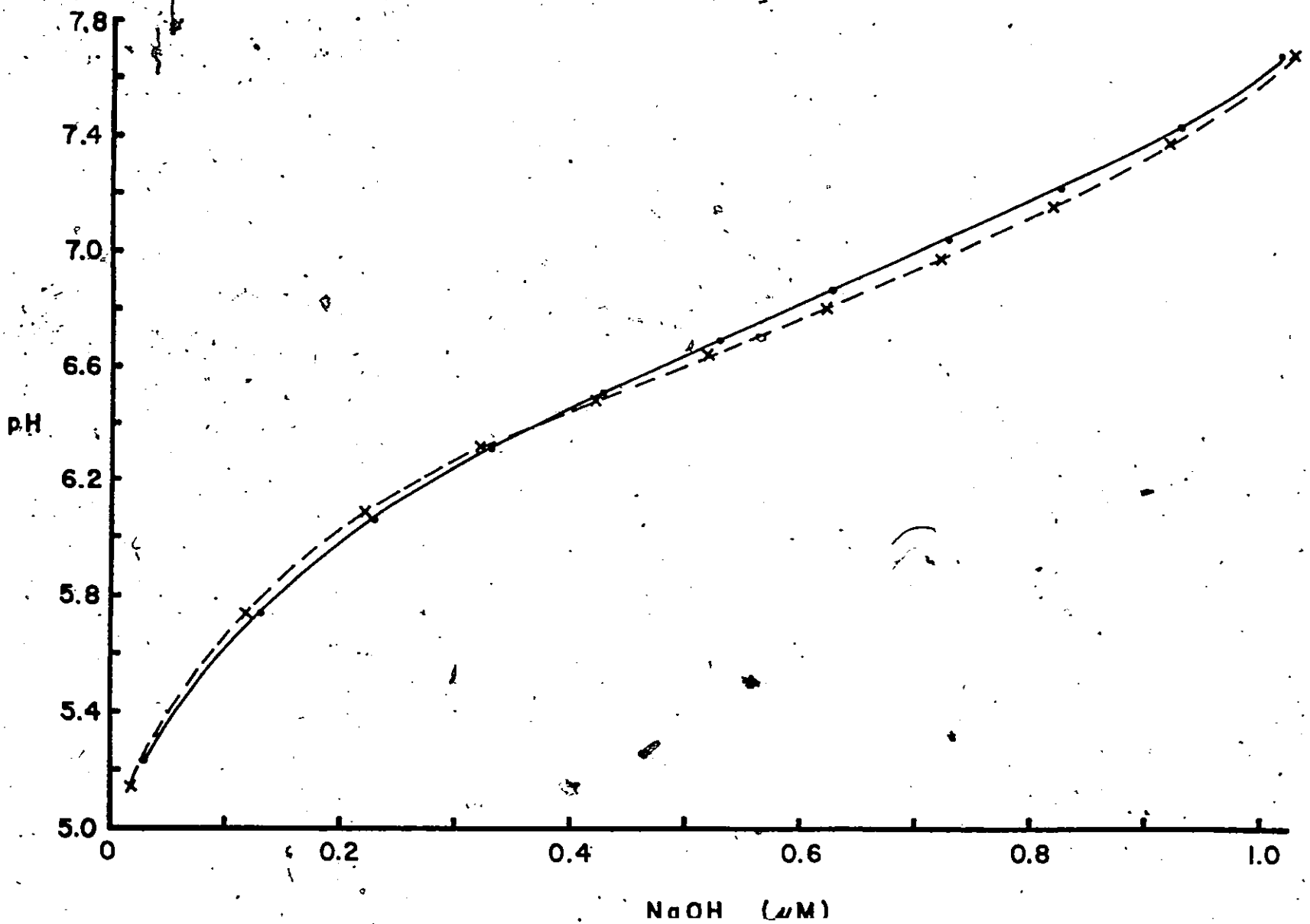
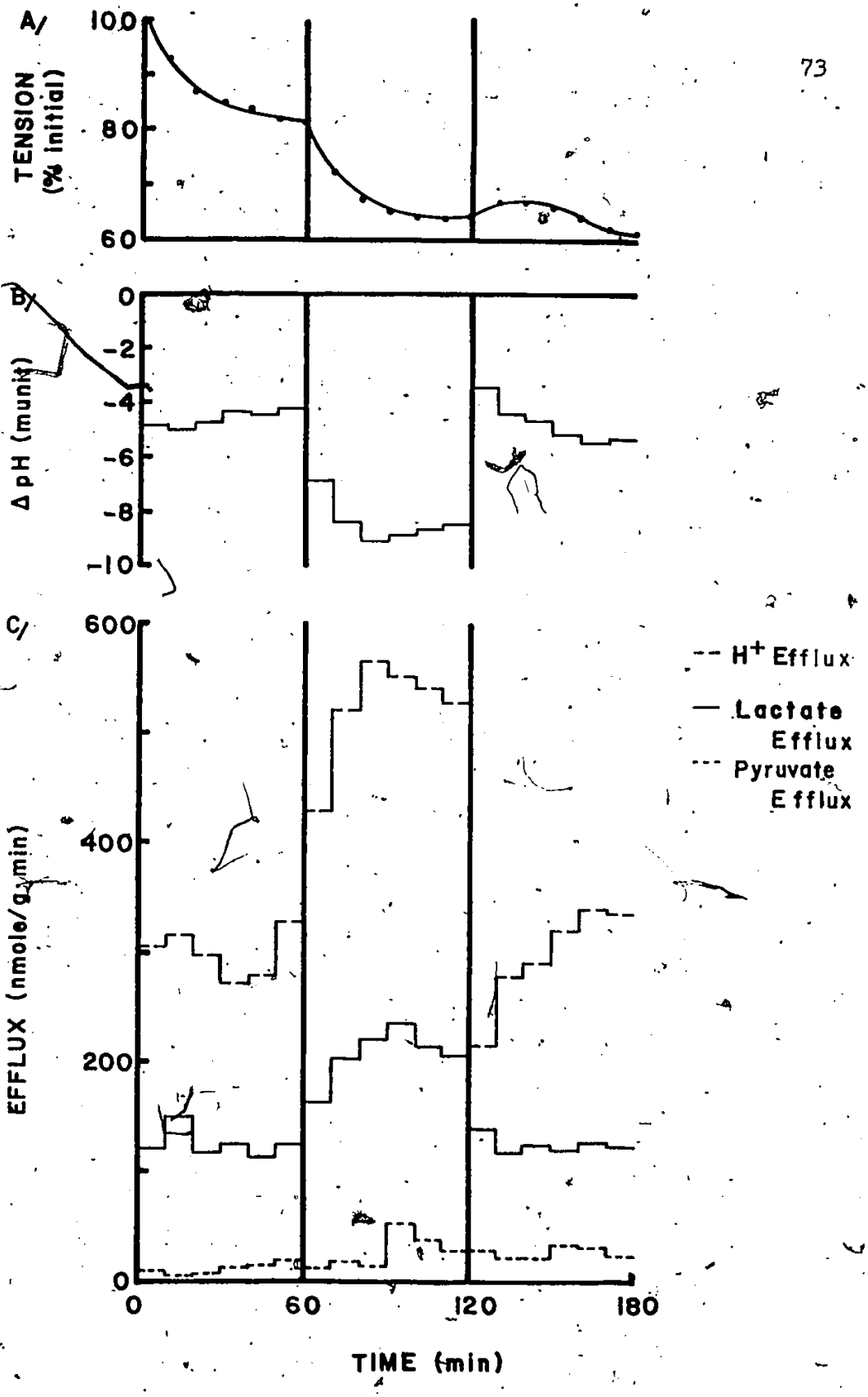

7.8
7.4
7.0
6.6
6.2
5.8
5.4
5.0
pH
0
0.2
0.4
0.6
0.8
1.0
NaOH (μM)

Fig. 10.

The results of a single experiment. The muscle was equilibrated in 1 ml Tyrode with a constant $[HCO_3^-]$ of 28 mM. The solution was changed every 10 minutes. The CO_2 level was initially 5%, lowered to 2% and returned to 5% with one hour at each pCO_2. Graph A illustrates tension changes as a percent of the initial tension (102 g). Graph B illustrates the change in the pH of the Tyrode equilibrated with the muscle. The H^+ efflux determined from the change in pH is shown in graph C which also shows the lactate and pyruvate effluxes as measured by enzyme assay.


A/
TENSION (% initial)
100
80
60
B/
ΔpH (munit)
0
-2
-4
-6
-8
-10
C/
EFFLUX (nmole/g.min)
600
400
200
0
0
60
120
180
TIME (min)
-- H+ Efflux
— Lactate Efflux
--- Pyruvate Efflux

experiment the muscle was maintained for 1 hour in normal Tyrode. During the second hour the CO_2 was decreased to 2% (pH 7.88) and finally returned to normal for the third 1 hour period.

Graph A illustrates the change in muscle tension which is expressed as a percent of the initial. The change to 2% CO_2 depresses tension approximately 15% while the return to normal elevates it only slightly. Initial maximum tetanus tension for the muscle was 102 g which yielded a normalized tension of 12.84 g/mm^2.

The second graph in fig. 10 shows the changes in pH for each 10 minute period. Under control conditions, each 10 minutes during which 1 ml of normal Tyrode was exposed to the muscle resulted in a shift in pH of -0.0045. During 2% CO_2 this change in pH increased to -0.009. Upon return to normal it went slightly less negative than the resting value,

then levelled off at about -0.005.

H^+ efflux was calculated from the change in pH for each interval using equation I (Appendix). Graph C illustrates the net H^+ efflux. Resting H^+ efflux was about 300 nM/g per minute. Following a change in CO_2 from 5% to 2% the H^+ efflux increased gradually to 550 nM/g per minute. Upon return to 5% CO_2 it was reduced to slightly below the initial efflux but gradually increased to a final value of about 350 nM/g per minute.

Lactate and pyruvate were measured directly by enzyme assay and the results are also shown in graph C of fig. 10. Lactate increases above the resting value of about 125 nM/g per minute to 225 nM/g per minute during 2% CO_2. The initial efflux level is restored by a return to 5% CO_2. Pyruvate efflux is small compared with that of lactate. A gradual increase in efflux occurs throughout the experiment.

C. The Effects of Varying CO_2 and Bicarbonate Concentration on Lactate and Pyruvate Efflux

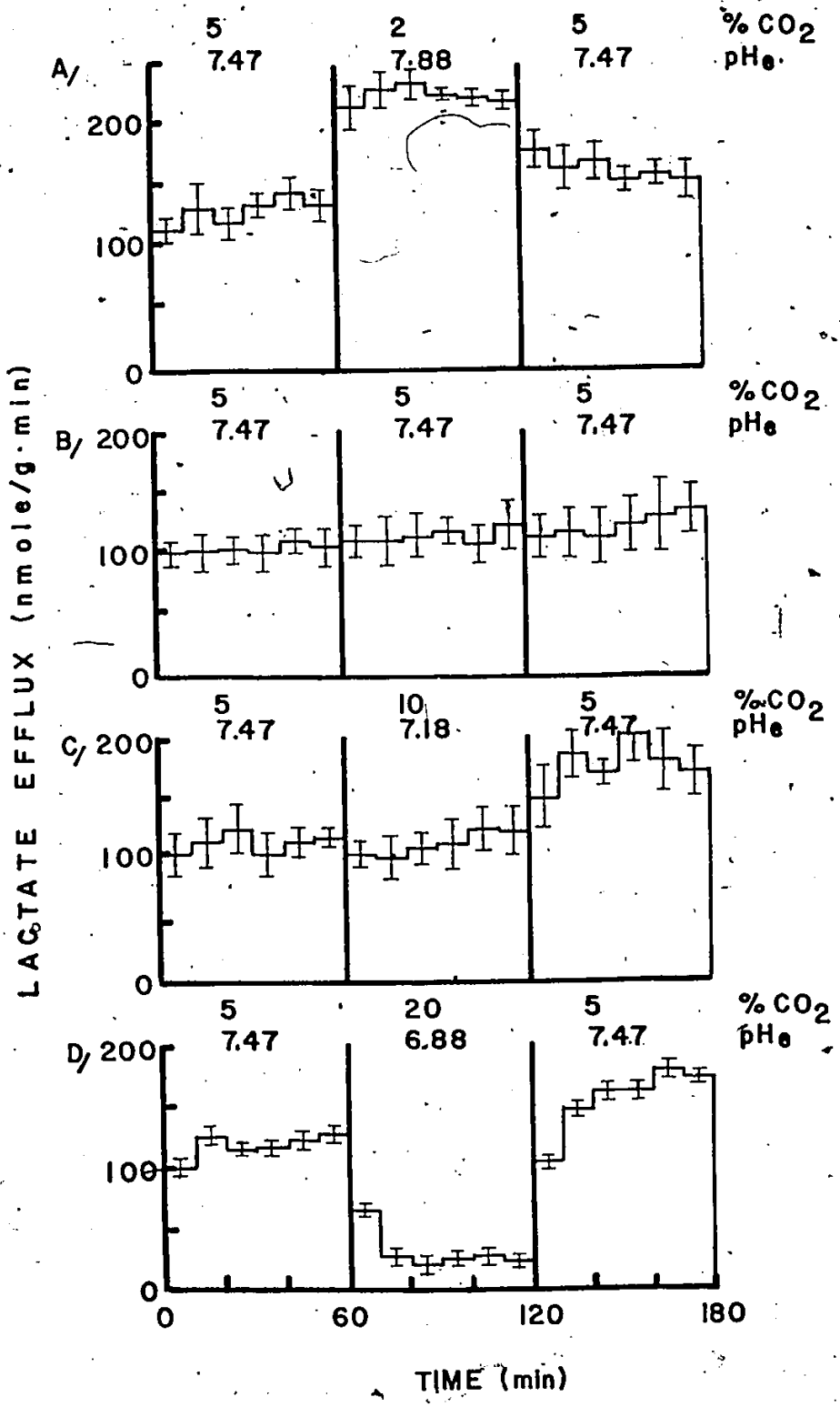
1). Effects of Changing CO_2 Concentration

The lactate efflux for a series of experiments in which the pCO_2 was changed with a constant bicarbonate concentration is illustrated in fig. 11. The controls in 5% CO_2 throughout the duration of the experiment showed no significant change in 3 hours time. However, a decrease in CO_2 from 5% to 2% resulted in an increased efflux of lactate. An increase to 10% CO_2 (graph C) did not result in any significant change in efflux although 20% CO_2 reduced the efflux to about one-quarter the initial value.

Return to normal CO_2 from both 10% and 20% CO_2 results in a gradual drift of the lactate efflux to a value greater than what was observed for resting conditions. Return to 5% from 2% CO_2 results in a gradual drift to a level that is expected for the third hour from

Fig. 11.

Changes in lactate efflux in response to different levels of CO_2. Values shown are ± SEM (n=4) in each set of experiments. Muscles were maintained in 1 ml Tyrode with a constant $[HCO_3^-]$ of 28 mM. The pH_e of the bathing solution is indicated for each CO_2 level.


A/
5
7.47
2
7.88
5
7.47
% CO2
pHe
200
100
0
B/
5
7.47
5
7.47
5
7.47
% CO2
pHe
200
100
0
C/
5
7.47
10
7.18
5
7.47
% CO2
pHe
200
100
0
D/
5
7.47
20
6.88
5
7.47
% CO2
pHe
200
100
0
0
60
120
180
TIME (min)
LACTATE EFFLUX (nmole/g·min)

the control experiment results.

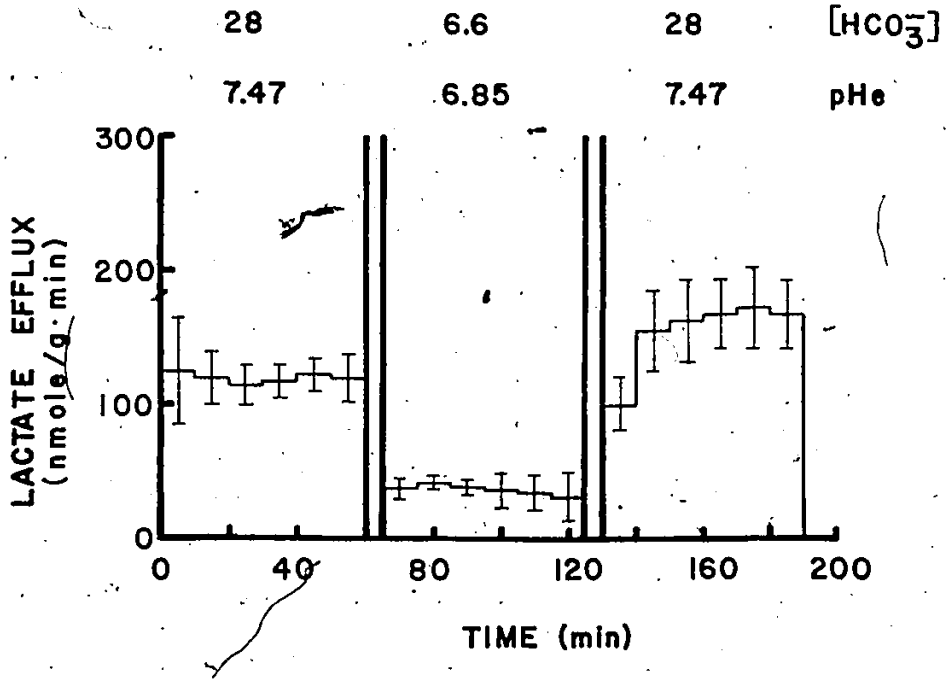
2. Effects of Changing Bicarbonate Concentration

One set of muscles was maintained in a constant pCO_2 solution while the $[HCO_3^-]$ in the bathing solution was changed to 6.6 mM at the end of the first hour. This change results in a pH_e (6.85) similar to the pH resulting from the equilibration of 20% CO_2 with 28 mM bicarbonate.

The changes in lactate efflux shown in fig. 12 are similar to those for the experiments in which the CO_2 was increased to 20% (fig. 11, D). In fig. 12 resting lactate efflux was about 120 nM/g per minute. After the change to 6.6 mM HCO_3^- it was immediately reduced to about 25% of the initial value. Return to normal pH_e resulted in a lactate efflux that was 50 nM/g per minute greater than the initial efflux in the same bathing solution.

Fig. 12.

Lactate efflux at constant pCO_2 during changes in external HCO_3^- concentration. The values indicated are the mean efflux of 4 experiments ± SEM. Muscles were maintained in 1 ml Tyrode at a constant level of 5% CO_2. The pH_e of the bathing solution is shown for each HCO_3^- concentration. The 5 minute breaks between the 1 hour periods were to allow time for a 1 ml rinse and 50 ml washout with the fresh HCO_3^- solution.


28
6.6
28
[HCO3-]
7.47
6.85
7.47
pHe
300
200
100
0
LACTATE EFFLUX
(nmole/g·min)
0
40
80
120
160
200
TIME (min)

3. Effects of Changing Both CO_2 and HCO_3^- Concentration

A single experiment was performed in which 20% CO_2 was superimposed on 6.6 mM HCO_3^-. The resulting pH_e was 6.24. The lactate efflux results for this experiment are illustrated in fig. 13. With each decrease in pH_e the lactate efflux was also diminished, until at the end of one hour in pH_e 6.24 it was virtually abolished. An overshoot of about 60% of the initial resting efflux occurs upon return to normal pH_e.

4. Variation in Lactate Efflux with External pH

Fig. 14 is a plot of lactate efflux against the pH of the bathing solution. Points A and B represent the pH_e obtained with 2% and 10% CO_2 and normal HCO_3^-. Points C_1 and C_2 are the results for 20% CO_2 and 6.6 mM HCO_3^- respectively. Point D at a pH_e of 6.24 is

Fig. 13.

Lactate efflux during HCO_3^- and CO_2 changes. The muscle began in normal Tyrode pH 7.47, underwent a decrease in HCO_3^- followed by a superimposed increase in CO_2. The pH was returned to normal in two steps by first restoring the CO_2 then the HCO_3^-. The muscle spent 1 hour in each pH_e with 5 minute, 50 ml washouts for each change in HCO_3^-.

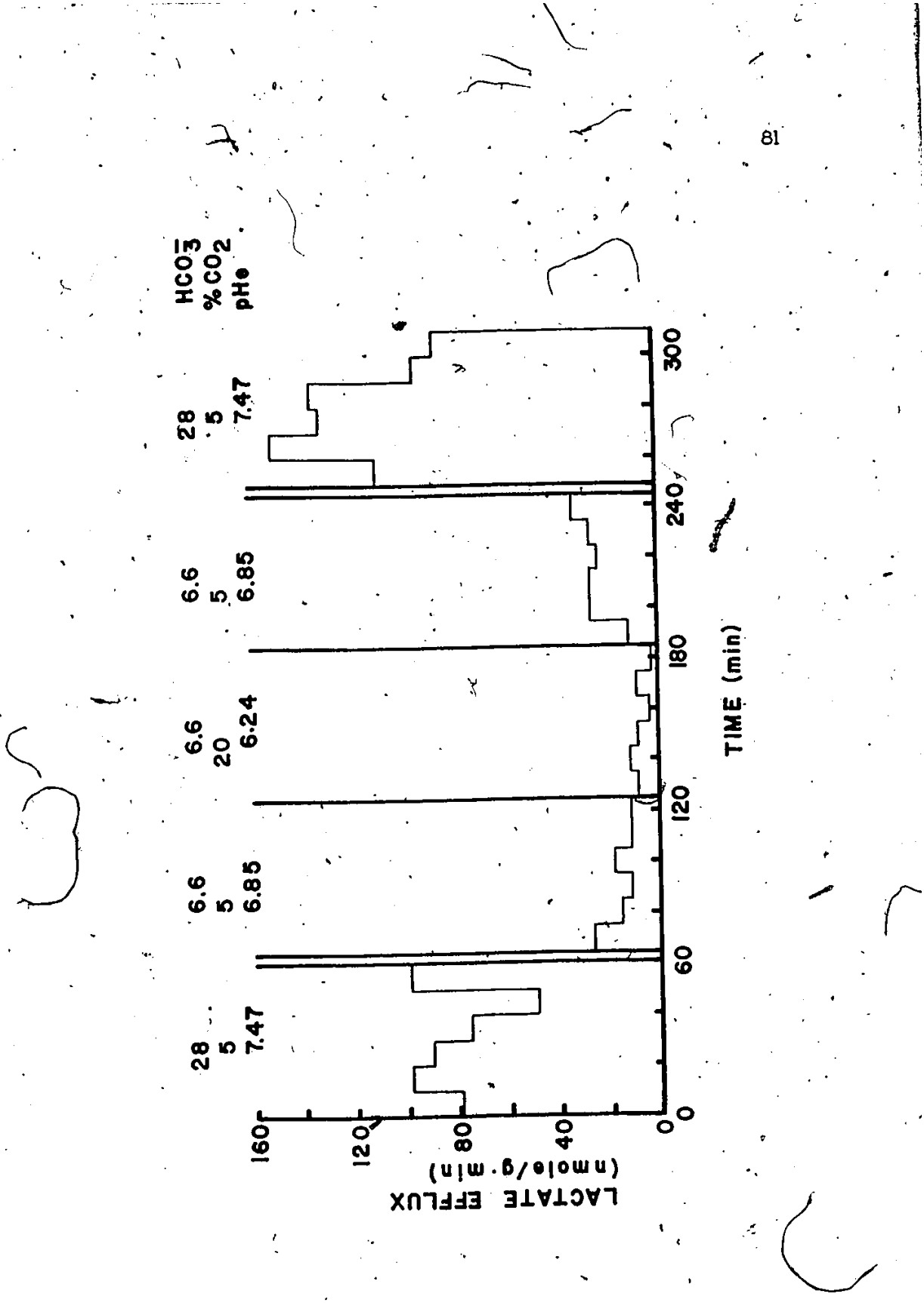
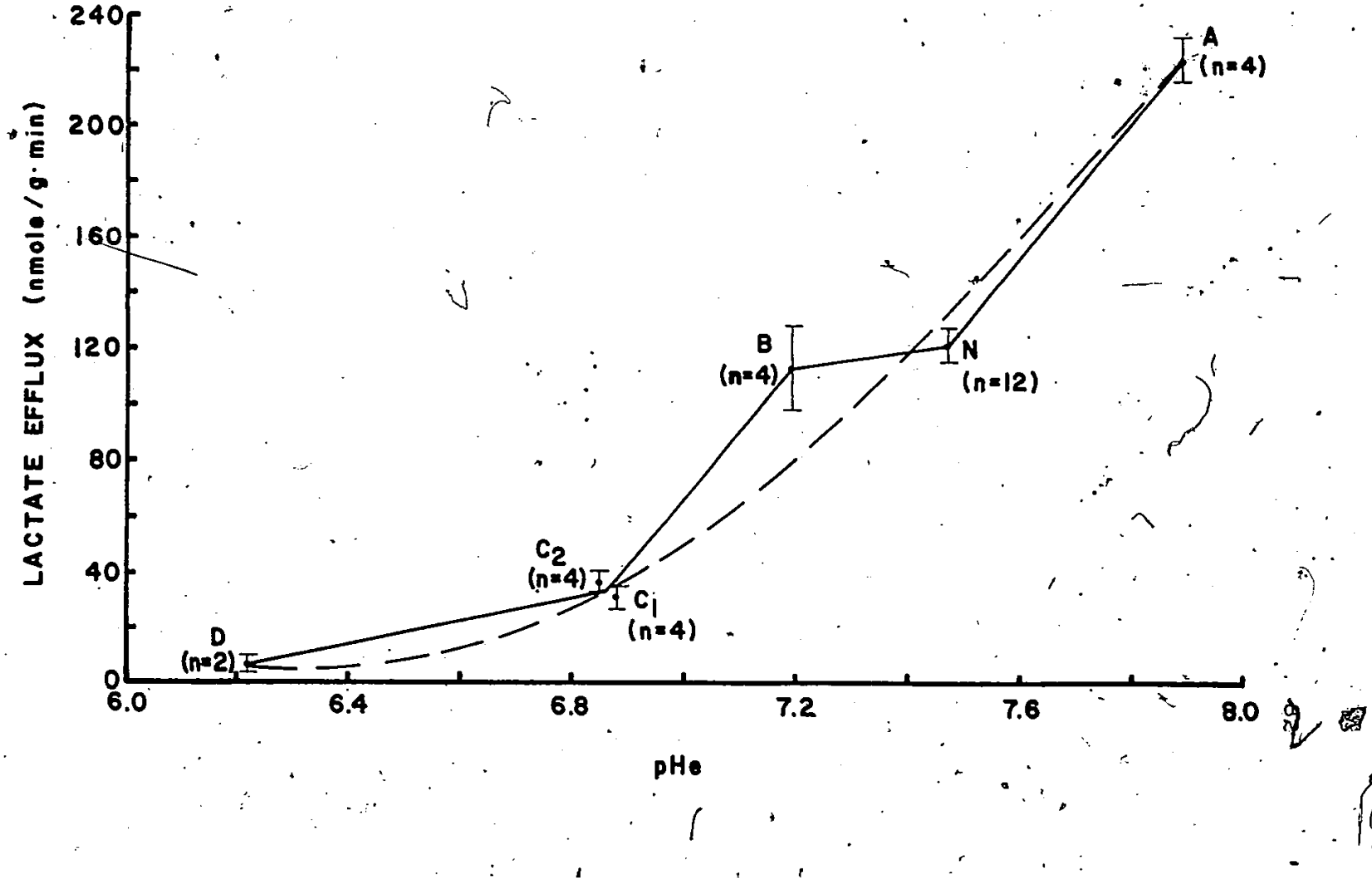

LACTATE EFFLUX
(nmole/g·min)
0
40
80
120
160
TIME (min)
0
60
120
180
240
300
HCO3
%CO2
pHe
28
5
7.47
6.6
5
6.85
6.6
20
6.24
6.6
5
6.85
28
5
7.47

Fig. 14.

Relationship between external pH and lactate efflux. Point N represents lactate efflux in normal Tyrode (28 mM HCO_3^- and 5% CO_2). The pH_e for points A and B are due to changes in CO_2 of 2% and 10% respectively. Points C_1 and C_2 are determined by results from 20% CO_2 and 6.6 mM HCO_3^- experiments respectively. Point D with a pH_e of 6.24 is from the results obtained by superimposing 20% CO_2 on 6.6 mM HCO_3^-. Each point is shown ± SEM, n is given in parentheses.


LACTATE EFFLUX (nmole/g·min)
240
200
160
120
80
40
0
6.0
6.4
6.8
7.2
7.6
8.0
pHe
A
(n=4)
B
(n=4)
N
(n=12)
C2
(n=4)
C1
(n=4)
D
(n=2)

the result of 20% CO_2 superimposed on a 6.6 mM HCO_3^- change. The graph indicates that lactate efflux is pH dependent and increases by more than 20 fold over the pH_e range 6.2 to 7.9.

There are two points which can be made about the characteristics of this graph. The first is that the solid lines connecting the individual points with a plateau between the points B and N is much the same as that of a plot of intracellular pH against extracellular pH for rat diaphragm from two other sources (Adler, Roy and Relman, 1972; Heisler, 1975). This might suggest some connection between lactate efflux and pH_i. However, it can be seen from fig. 11 and fig. 12 that the decrease in lactate efflux was as quick or quicker in response to a decrease in HCO_3^- as it was in response to an increase in pCO_2. One might expect that a decrease in HCO_3^- would result in a slower

change in pH_i than an increase in pCO_2 due to the readiness with which CO_2 crosses the membrane.

The second point is that the curved, dashed line in fig. 14 can be compared favourably with the shape of the graphs obtained by Hutter and Warner (1967) and Woodbury and Miles (1973) relating chloride conductance to extracellular pH. This concurs with the idea that lactate behaves as a chloride-like anion (Woodbury and Miles, 1973).

5. Pyruvate to Lactate Ratios

Table II illustrates the changes in the pyruvate to lactate (P/L ratio). The control values indicate that during resting conditions this ratio increases as the experiment progresses. The experiments in which CO_2 was changed to 2% deviated little from the control values. An increase to 10% CO_2 also increased the P/L

TABLE II

PYRUVATE EFFLUX AS A PERCENT OF LACTATE

Experiment	Time 0-60	60-120	120-180 minutes
Control	11.8±1.36 (n=12)	13.7±1.30 (n=11)	19.4±2.18 (n=11)
Constant HCO_3^-			
5-2-5% CO_2	10.8±1.24 (n=12)	13.8±1.48 (n=12)	22.5±0.89 (n=11)
5-10-5% CO_2	10.4±0.96 (n=18)	17.7±0.98 (n=18)	14.6±0.89 (n=18)
5-20-5% CO_2	8.2±1.71 (n=3)	--	10.3±0.46 (n=3)
Constant CO_2			
28-6.6-28 mM HCO_3	4.8±1.52 (n=4)	12.1±5.68 (n=4)	12.9±2.16 (n=2)

ratio. Return to normal from 10% lowered the P/L ratio.

No value is given for the P/L ratio following a change to 20% CO_2. Lactate efflux in this situation was reduced to about 30 nM/g per minute (fig. 11, D). The lowest sensitivity at which pyruvate efflux could be measured was about 5 nM/g per minute for an average-sized muscle. Therefore, an accurate measurement of pyruvate efflux during 20% CO_2 could not be obtained unless the P/L ratio was greater than 30%.

The P/L ratio for the three periods of the experiments in which bicarbonate concentration was altered do not significantly differ from the trend that is predicted in the control experiments.

6. Intracellular Lactate Results

Table III gives the results of some intracellular lactate measurements made on muscles

TABLE III INTRACELLULAR LACTATE RESULTS

Conditions	Lactate Concentration (uM/g wet wt)
A	
90 min in normal + 15 min in 6.6 mM HCO_3^-	2.97 ± 0.593 (n=6)
B	
105 min in normal	3.62 ± 1.031 (n=6) *
C	
90 min in normal + 65 min in 6.6 mM HCO_3^-	3.32 ± 1.557 (n=6)
D	
155 min in normal	2.69 ± 0.661 (n=6) *

A and C in low 6.6 mM HCO_3^- were preceded by 90 minutes in normal bicarbonate to approximate the conditions in the experiments where acid base exchange and lactate efflux were measured. Results are followed by standard deviations.

* B and D are significantly different (< 0.05) according to "t" test for unpaired variables.

which were exposed to pH_e changes in the bathing solution due to a decrease in bicarbonate concentration. The results of controls are included which were exposed to Tyrode solution for the same length of time without a change in pH_e.

The control results indicate that there is a significant decrease in the intracellular lactate level with time. However, the results for the muscles exposed to 6.6 mM HCO_3^- do not show this decrease. Therefore, the low bicarbonate seems to prevent the expected decrease in muscle lactate.

D. The Effect of Varying CO_2 and Bicarbonate Concentration on Acid Base Exchange

1. Effects of Changing CO_2 Concentration

If the assumption is made that lactate and pyruvate are transferred through the membrane in their ionic form, then movements of H^+ will be independent of the transfer of the metabolic

anions. Therefore, I would first like to consider total net acid efflux separately from lactate and pyruvate efflux.

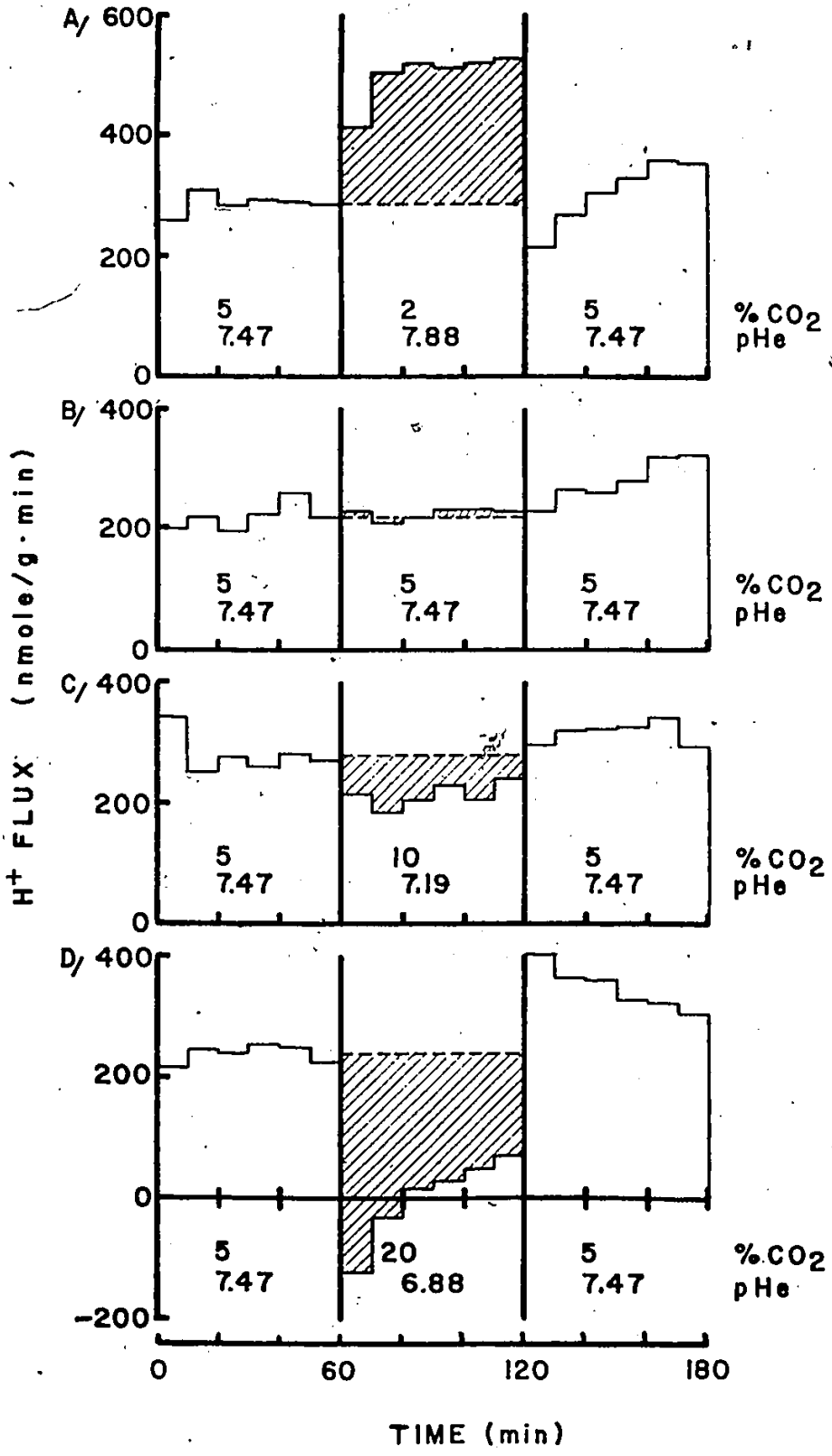
The total net acid efflux for the experiments in which CO_2 was altered is shown in fig. 15. The H^+ efflux for the control experiments remained stable throughout the 3 hours duration at a level of approximately 250 nM/g per minute. Towards the end of the third hour a small increase appeared which is not statistically significant. Two experiments were continued for five hours duration. They showed that this increase does not continue to rise to a significant level.

During 2% CO_2 the net acid efflux increased gradually to about 525 nM/g per minute from 290 nM/g per minute. Return to 5% CO_2 resulted initially in values lower than the resting level with a gradual return to normal efflux.

Conversely, an increase in pCO_2 results

Fig. 15.

Changes in H^+ flux in response to different levels of CO_2 with a constant HCO_3^- concentration of 28 mM. Each graph represents the mean of 4 experiments with an average SEM of ±25 nM/g per minute. Dashed lines during the second hour are the average of the values during the first hour under control conditions. Therefore, the sum of the shaded area represents the total base exchange for 1 hour after a change in pCO_2. The values for the sum of each area are: 2% - 12.79 mM/kg; 10% - 3.93 mM/kg; and 20% - 14.02 mM/kg.


A/
B/
C/
D/
600
400
200
0
-200
5
7.47
2
7.88
10
7.19
20
6.88
%CO2
pHe
H+ FLUX (nmole/g·min)
0
60
120
180
TIME (min)

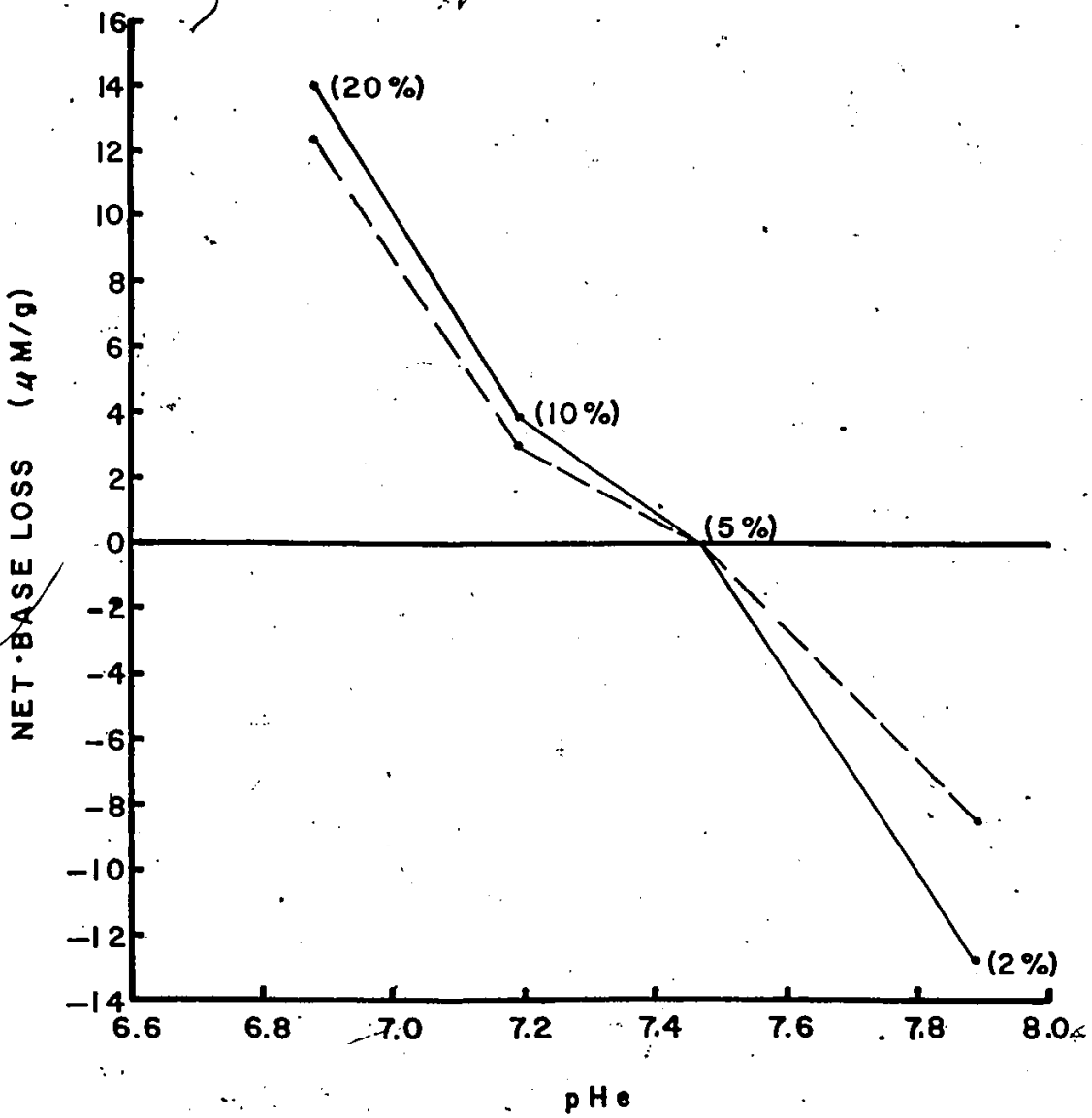
in a decrease in net acid efflux and actually leads to a temporary reversal in efflux for the 20% CO_2. After 20 minutes in 20% the net acid efflux again returns to a positive value and at the end of one hour has reached a value one third that of resting conditions. Return to 5% CO_2 from 20% results in an overshoot of the initial efflux with a gradual return to the resting condition level.

An average value of the resting acid efflux for the first six 10 minute periods in each set of experiments can be used to predict the net acid efflux expected during the second hour if there were no changes in pCO_2. Any deviation from this value can represent the transfer of HCO_3^- (H^+ or OH^-) in response to the change in CO_2. Therefore, the sums of the shaded areas in fig. 15 represent the quantity of HCO_3^- (H^+ or OH^-) transferred in one hour due to a change in pCO_2.

In fig. 16 the sum of each area is

Fig. 16.

Changes in net base loss with respect to pH_e. My results (solid line) represent values determined from the change in net acid efflux from the resting value integrated over a one-hour period following a change in pCO_2. The percent CO_2 used to obtain each pH_e is indicated in parentheses beside each point. Values, calculated from Heisler's (1975) results are also graphed (dashed line). These values were determined by taking the difference between the total bicarbonate generated in a closed system (muscle homogenates) and the intracellular bicarbonate concentration values estimated by the DMO method in intact muscle after equilibration at different CO_2 levels while allowing acid base exchange with the bathing medium.


16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
NET-BASE LOSS (μM/g)
(20%)
(10%)
(5%)
(2%)
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pHe

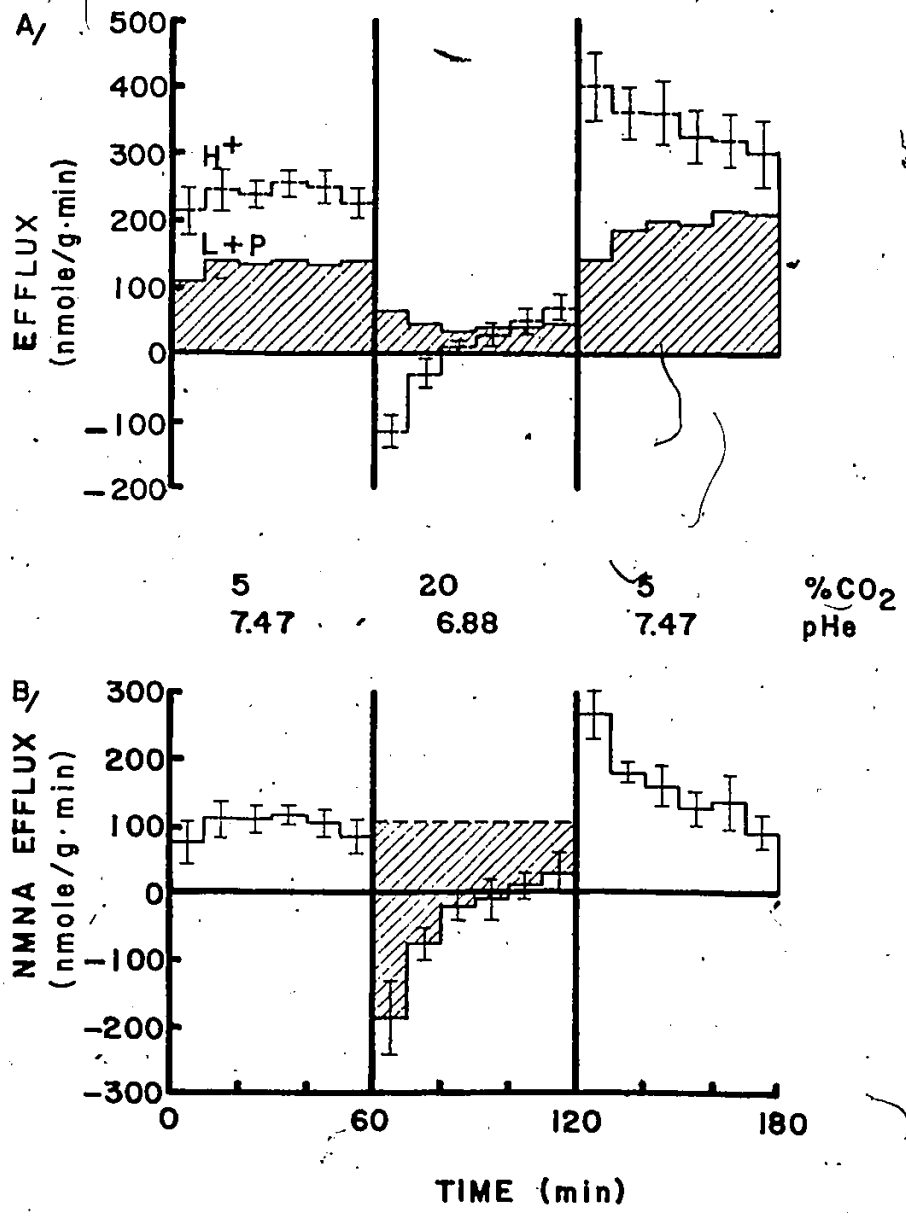
plotted against extracellular pH. From pH_e 6.88 to 7.89 net base loss varies from 14 mM/kg muscle to -12.8 mM/kg muscle. However, the graph is not linear. There appears to be a plateau area in the pH_e range from 7.5 to 7.2 during which the net base loss decreases less steeply with an increase in pH_e.

Thus far, we have been concerned with the total net acid efflux based on the assumption that the transfer of lactate and pyruvate is in the anionic form and does not contribute to the measured net acid efflux results. For comparison, I have included one set of results (fig. 17 A) for the 20% CO_2 experiments in which the lactate plus pyruvate effluxes and total net acid are graphed together. As previously indicated, the resting lactate plus pyruvate efflux is about two-thirds that of the total net acid efflux during resting conditions. The rest of the graph results are as previously illustrated on separate graphs (fig. 11, D and fig. 15, D).

Fig. 17.

A The effect of a change to 20% CO_2 on the net H^+ efflux and lactate + pyruvate efflux. Mean net H^+ flux is indicated by the solid line (4 experiments) ± SEM. The mean lactate + pyruvate is shown by the shaded area (L+P).

B The effect of a change to 20% CO_2 on the non-metabolic net acid (NMNA) efflux. Values derived from the above graph by subtracting L+P efflux from the net H^+ efflux. Values are the mean of 4 experiments ± SEM. Dashed line in the second hour represents the average efflux during resting conditions from the first hour. Therefore, the sum of the shaded area represents total base exchange during the first 60 minutes following the change to 20% CO_2. The value for this area is 8.53 mM/kg muscle.


A/
500
400
300
200
100
0
−100
−200
EFFLUX
(nmole/g·min)
H+
L+P
5
7.47
20
6.88
5
7.47
%CO2
pHe
B/
300
200
100
0
−100
−200
−300
NMNA EFFLUX
(nmole/g·min)
0
60
120
180
TIME (min)

If the assumption is made that lactate and pyruvate cross the membrane as the undissociated acid then it is necessary to consider the acid efflux as the total net acid efflux minus the H^+ transferred in the lactic and pyruvic acid efflux. These values of total net acid efflux minus the lactate plus pyruvate efflux will be referred to as the non-metabolic net acid (NMNA) efflux. Fig. 17, B illustrates this for the results of the 20% CO_2 experiments. Compared with the total net acid efflux, the NMNA efflux results in a more pronounced reversal when changing to 20% CO_2. There is also a larger overshoot of the initial efflux immediately after return to normal for the NMNA efflux results. The net base loss determined from the NMNA results (shaded area fig. 17, B) is 8.53 mM/kg muscle compared with the 14.02 mM/kg muscle value obtained from the total net acid efflux (shaded area

15, D).

The NMNA efflux results from the other experiments in which CO_2 was changed, also predict less base exchange than the total net acid efflux results. The 2% CO_2 and 10% CO_2 experiments measured net base loss of -5.87 and 3.36 mM/kg muscle from NMNA efflux results, compared with -12.79 and 3.93 mM/kg muscle from total net acid efflux results.

2. Effect of Changing Bicarbonate Concentration

It was essential when changing to a different bicarbonate concentration that all the previous Tyrode solution be washed out of the muscle bath, since the high or low HCO_3^- concentration in the residual solution would be measured as a net H^+ change. A five minute, 50 ml washout did not remove all the previous solution, therefore, a ^{14}C-sucrose label in the initial Tyrode solution

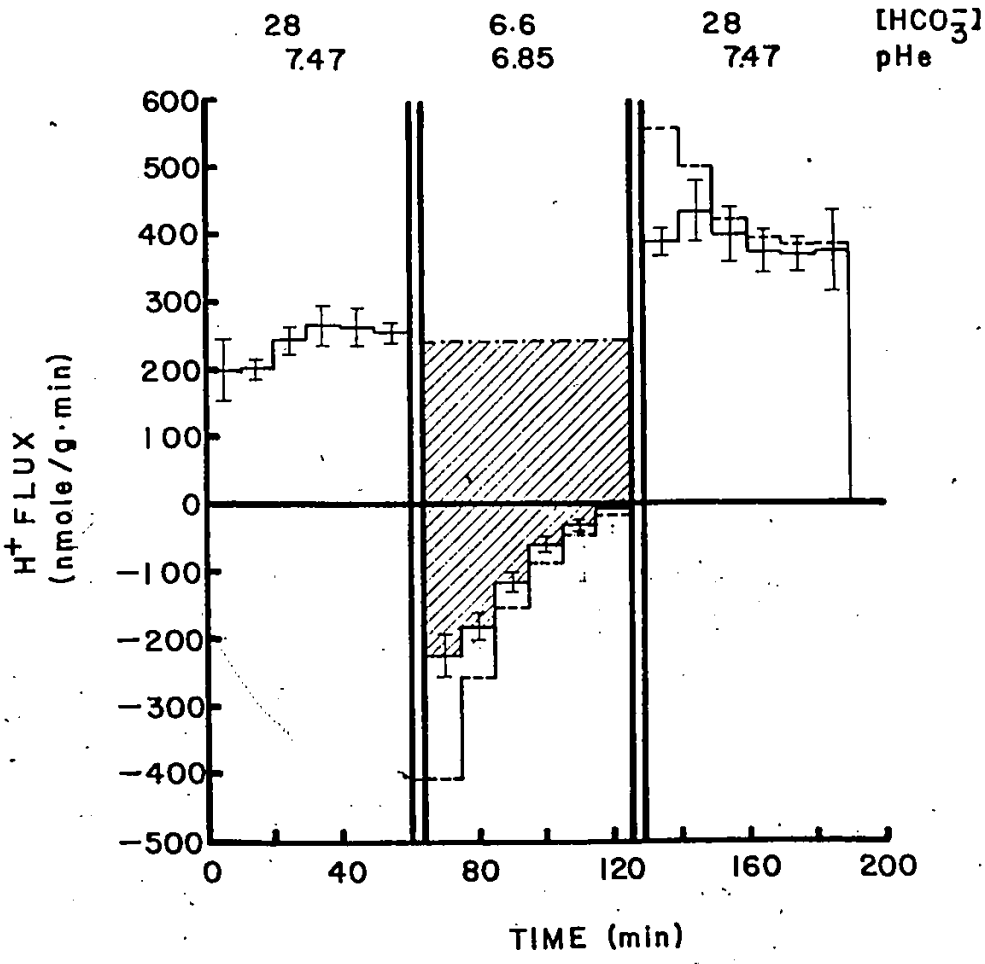
was used to determine the amount remaining. As indicated in Appendix B, the ratio of the ^{14}C-sucrose CPM's in each sample following the washout, to that in the labelled Tyrode indicated the proportion of the old solution mixed with the new. Using this ratio a corrected change in pH could be calculated.

In fig. 18 the net acid efflux is plotted for both the measured change in pH and after correcting with the ^{14}C-sucrose washout. The values represent the mean of 4 experiments. The SEM (not shown) for both the corrected and uncorrected averages 26.9 and 28.1 nM/g per minute respectively. As expected, the largest correction occurs in the first period following a change in HCO_3^-, and gradually decreases with each succeeding change in solution.

The pattern of change in net acid efflux following a decrease in HCO_3^- concentration to 6.6 mM is similar to an increase

Fig. 18.

H^+ flux during changes in HCO_3^- concentration at a constant pCO_2. Solid line is the net H^+ flux ± SEM (n=4). Dashed line (- -) is the H^+ flux uncorrected for the washout of extracellular HCO_3^-. Dashed and dotted line (-·-·-) during the second hour is the average of the H^+ efflux during the control period in the first hour. The sum of the shaded area represents the total base exchange during the first hour after the decrease in $[HCO_3^-]$. The sum of this area is 20.47 mM/kg muscle.


28
7.47
6.6
6.85
28
7.47
[HCO3-]
pHe
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
H+ FLUX
(nmole/g·min)
0
40
80
120
160
200
TIME (min)

in CO_2 to 20% (fig. 15, D). However, the initial reversal following the HCO_3^- concentration change is about 80 nM/g per minute more negative than the reversal brought about by the CO_2 increase. At the end of one hour in low bicarbonate the net acid efflux has not yet reached a positive value.

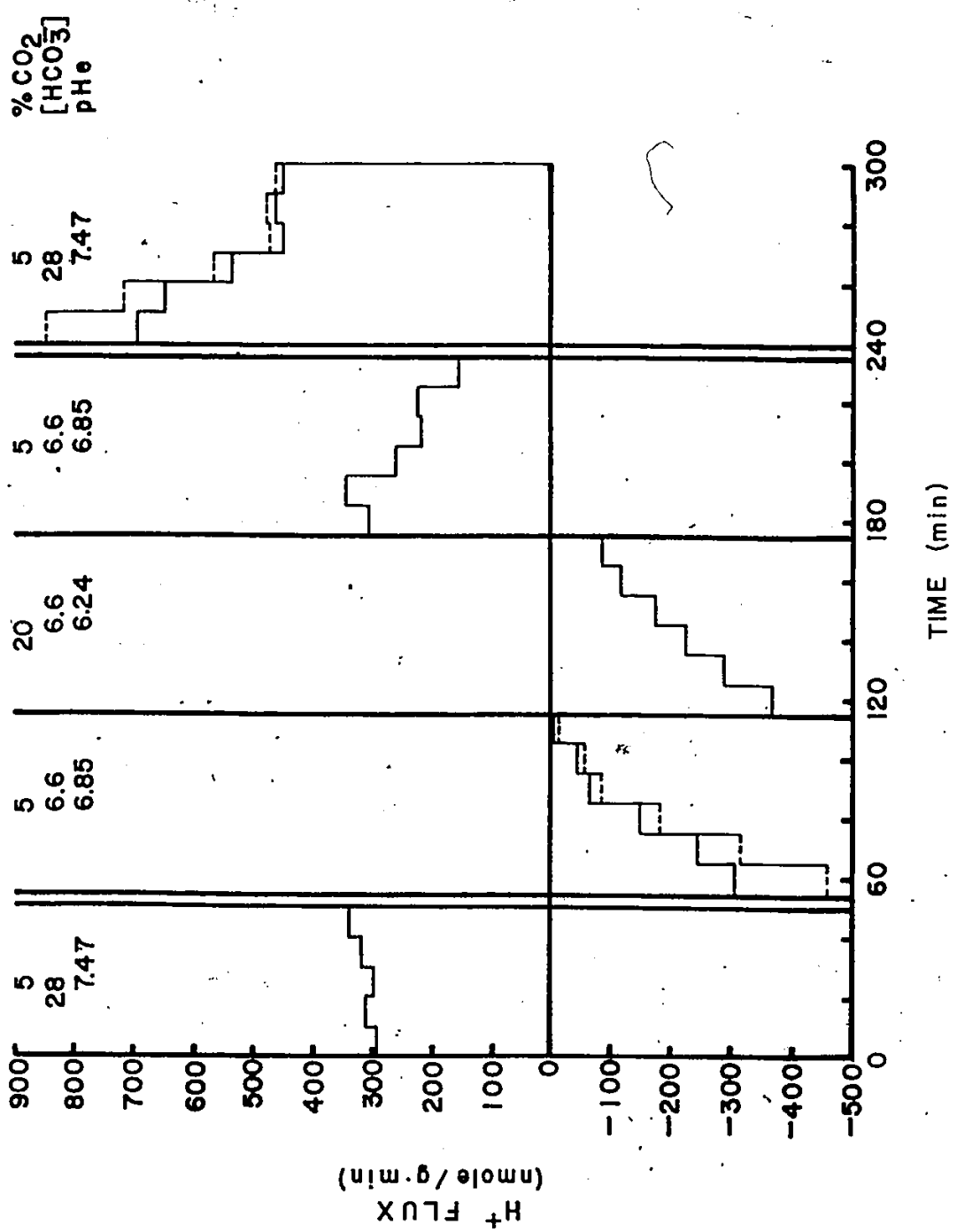
The shaded area in fig. 18 represents the total base exchange during the first hour after the decrease in HCO_3^- concentration. The sum of this area is 20.5 mM/kg muscle.

3. Effects of Changing Both Bicarbonate and CO_2 Concentration

The net acid efflux for the double change experiment is shown in fig. 19. The initial resting efflux and the changes in the second hour, are as expected for a bicarbonate change from normal to 6.6 mm HCO_3^-. When the high 20% CO_2 is superimposed on the low bicarbonate a further negative displacement in

Fig. 19.

Experiment in which H^+ flux is measured following changes in both CO_2 and HCO_3^-. The changes in the HCO_3^- concentration and CO_2 level are indicated at the top along with the corresponding pH_e. The dashed lines (---) are the H^+ flux uncorrected for the washout of extracellular HCO_3^- when there is a change in the HCO_3^- of the bathing solution.


%CO2
[HCO3-]
pHe
5
28
7.47
5
6.6
6.85
20
6.6
6.24
5
6.6
6.85
5
28
7.47
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
H+ FLUX
(nmole/g·min)
0
60
120
180
240
300
TIME (min)

net acid efflux is observed.

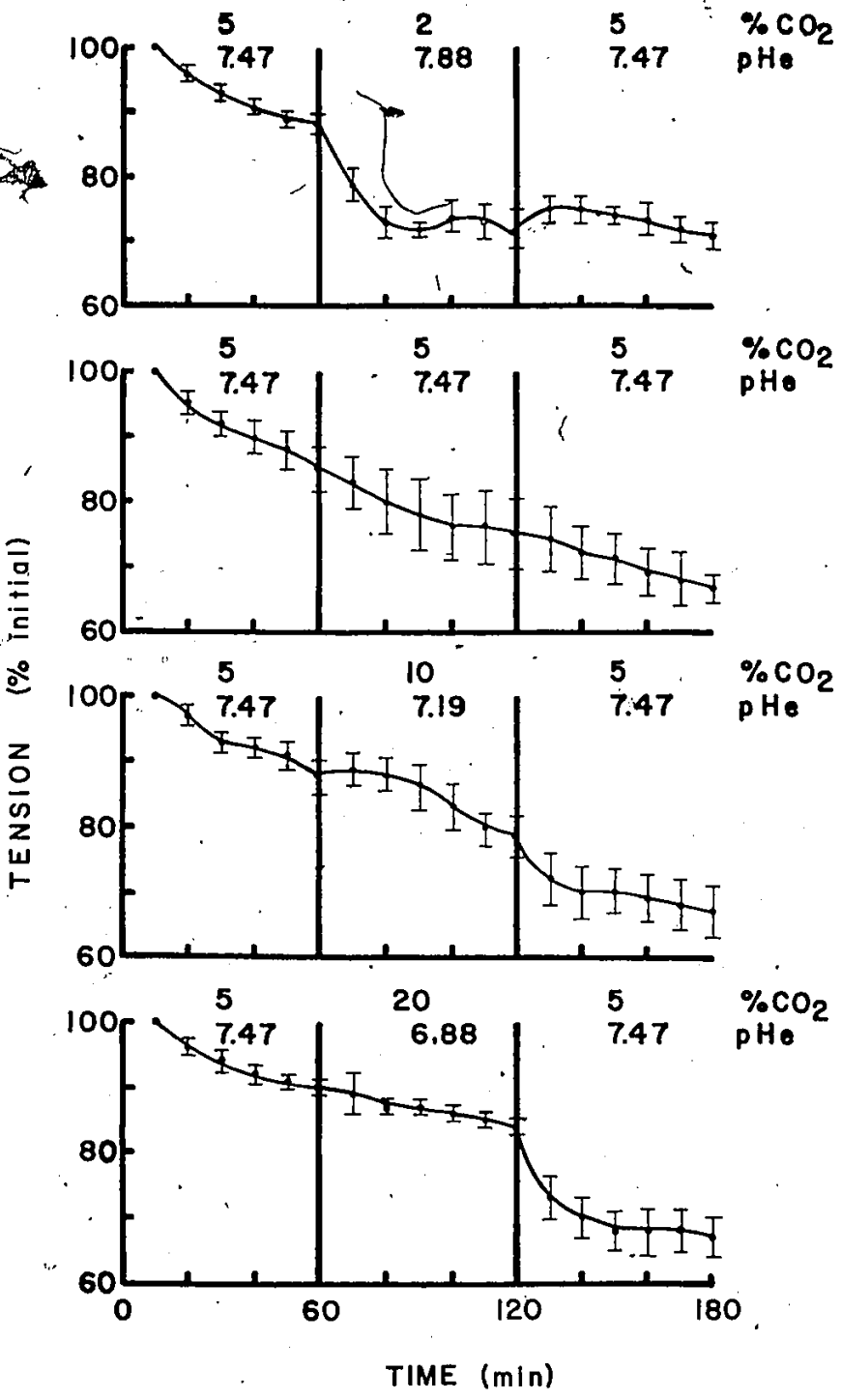
Returning to 5% CO_2 while maintaining the low bicarbonate restores a positive efflux to about the initial basal value although it gradually decreases to half that at the end of the hour. Complete restoration of normal pH_e results in a large overshoot which is more than double the initial resting value. There is some decrease in this value with time, although at the end of one hour it is still 50% above the initial efflux.

E. The Effects of Varying CO_2 and Bicarbonate Concentration on Muscle Tension

As indicated in the section on methods, the tetanic tensions are graphed as a percent of the initial tension which was taken as the tension at the end of the first 10 minute period in each experiment. The control experiments (fig. 20 A) indicate that tension loss is about

Fig. 20.

Effect of change in CO_2 on tension. For uniformity all experimental tensions were expressed as a percent of the initial tension which was taken as the tension at the end of the first 10 minute period. Muscles were stimulated with platinum electrodes at 1 tetanus every 200 seconds. Each point represents the values ±SEM (n=4) of the tetanic tension at the end of each 10 minute period.


5
7.47
2
7.88
5
7.47
%CO2
pHe
5
7.47
5
7.47
5
7.47
%CO2
pHe
5
7.47
10
7.19
5
7.47
%CO2
pHe
5
7.47
20
6.88
5
7.47
%CO2
pHe
100
80
60
TENSION (% initial)
0
60
120
180
TIME (min)

0.2%/minute over a 3 hour period.

In all cases, a decrease in pCO_2 resulted in a decrease in tension. This was true whether or not it was the result of starting at a normal CO_2 concentration followed by a decrease as well as returning to normal pCO_2 from an abnormally high level (fig. 20 B,C,D).

There does not seem to be an obvious enhancing effect of an increase in CO_2 concentration on muscle tension. However, if the values for muscle tension from the experiment involving increases to 10% and 20% CO_2 are examined 30 minutes after the CO_2 change and compared to control values it will be seen that their values are significantly higher (fig. 20, C and D). The return to 5% CO_2 from 2% CO_2 also results in an increase in muscle tension.

Bicarbonate concentration changes have an effect opposite to those of CO_2

concentration changes in that a decrease in $[HCO_3^-]$ results in a decrease in muscle tension (fig. 21). Return to a normal bicarbonate restores some of the tension loss.

The decrease in tension associated with a lowered bicarbonate concentration is observed in the first change in fig. 22. However, when a 20% CO_2 change is superimposed on the low bicarbonate a further decrease (~40%) occurs. This can be contrasted with the slight increase observed in fig. 20 D in response to an increase in CO_2. Returning to 5% CO_2 initially results in a slight increase (3%) in tension followed by a 14% decrease. Complete return to normal conditions restores only 15% of the tension.

F. Experiments Involving Changes in Activity and Anoxia

Fig. 21.

Effect of changes in HCO_3^- concentration on tension at constant pCO_2. Results are shown ±SEM (n=4) at the end of each 10 minute period. Tension is expressed as a percent of the initial

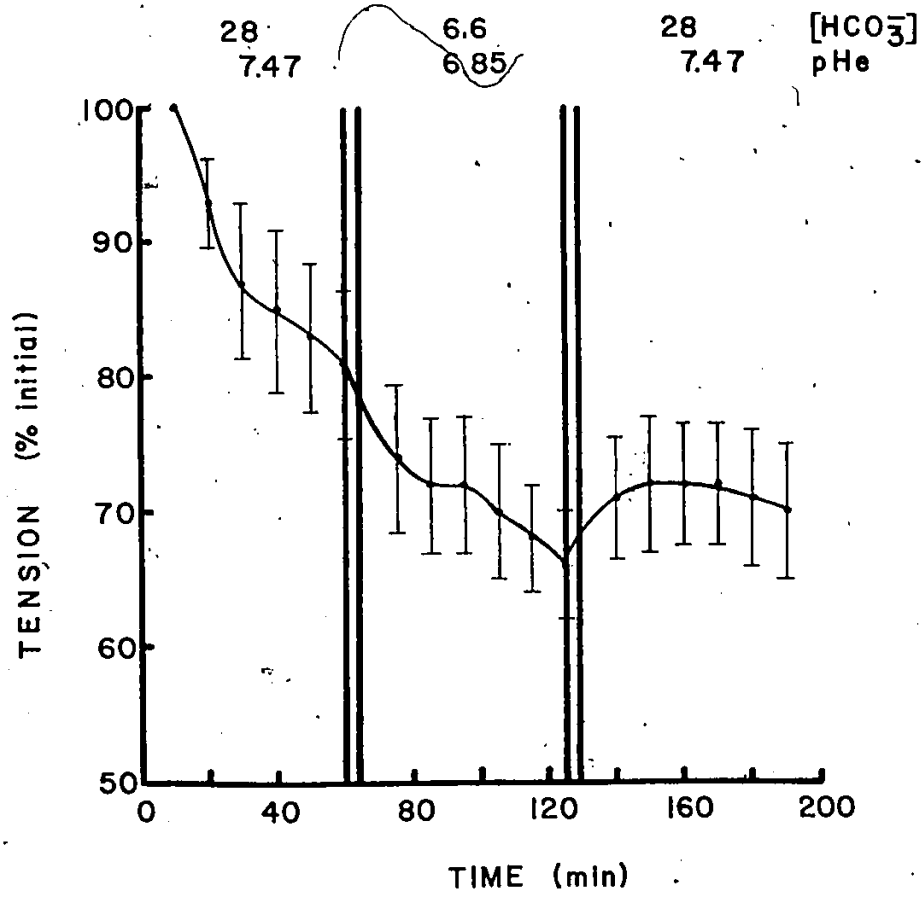
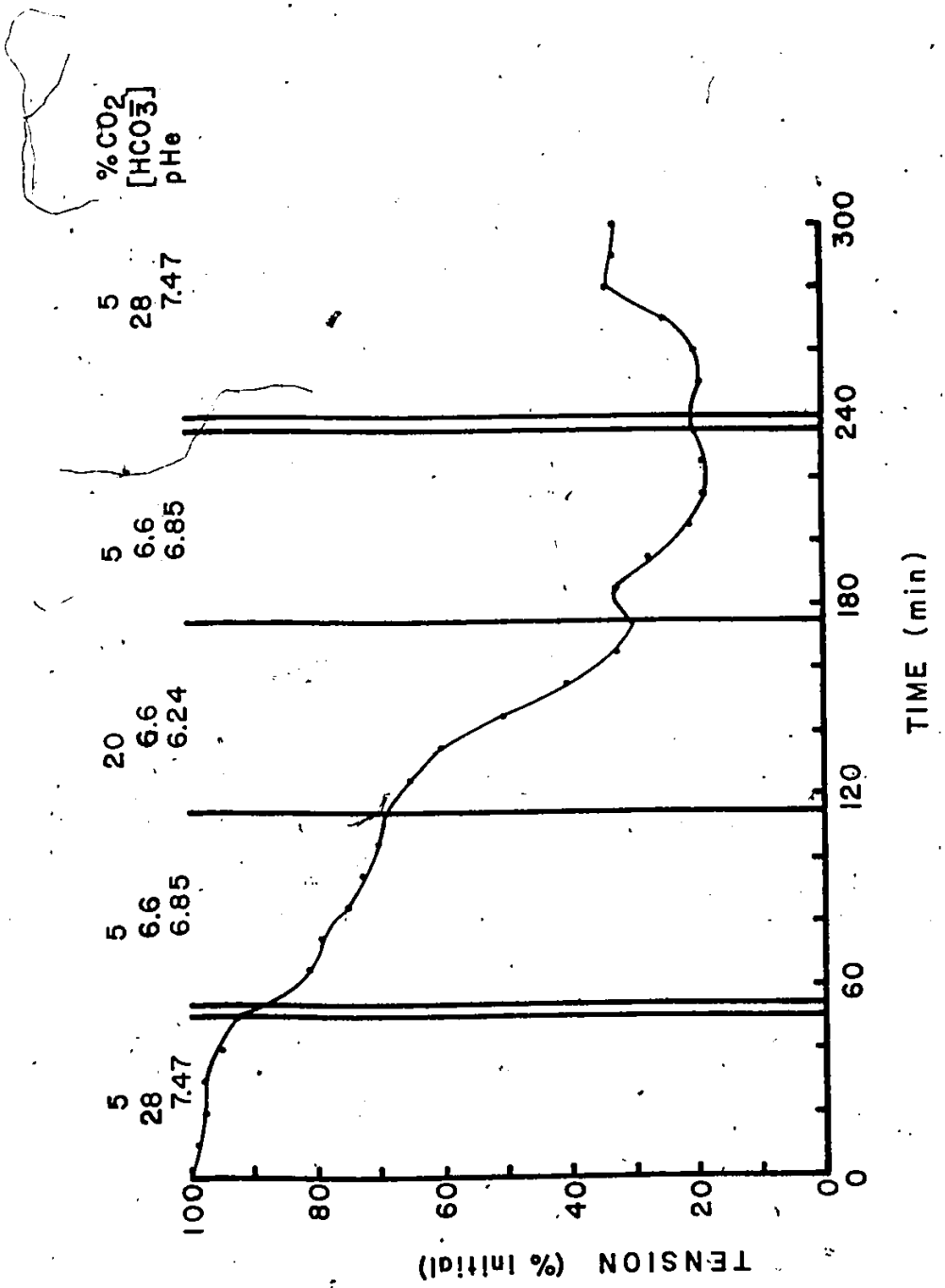

28
7.47
6.6
6.85
28
7.47
[HCO3-]
pHe
100
90
80
70
60
50
TENSION (% Initial)
0
40
80
120
160
200
TIME (min)

Fig. 22.

Effect of changing both HCO_3^- and CO_2 on tension. The graph is from the results of one experiment. The HCO_3^- concentration, CO_2 level and pH_e are indicated in the 3 lines at the top of the graph. Tension is expressed as a percent of the initial.


%CO2
[HCO3]
pHe
5
28
7.47
5
6.6
6.85
20
6.6
6.24
5
6.6
6.85
5
28
7.47
TENSION (% initial)
100
80
60
40
20
0
0
60
120
180
240
300
TIME (min)

1. Effects of Varying Stimulation on Lactate Efflux, H^+ Efflux and Muscle Tension

Resting lactate and net acid efflux in all the experiments previously described were made while the muscles were undergoing a brief ~0.2 second tetanus stimulus at 200 second intervals. These effluxes were not significantly different from when the muscle was maintained without any stimulation. Therefore, this stimulation rate was used as a measure of changes in tetanic tension in response to varying CO_2 and HCO_3^- concentrations in those experiments. However, I was also interested in the lactate and net acid efflux changes which could be elicited through increases in stimulation rates causing increased activity of the muscle.

When the muscle was stimulated by impulse trains at 5 second intervals for a 10 minute period, the resting lactate efflux

increased to 325 nM/g per minute (fig. 23). During the first 10 minutes of recovery the lactate efflux was even higher, reaching a level of 400 nM/g per minute. Other stimulation rates at 50 second and 10 second intervals resulted in increases in lactate efflux of 90% and 150% respectively over resting condition levels (fig. 24).

As indicated in the introduction, there has been much controversy over whether or not the H^+ leaves the muscle wall with the measured lactate changes. Due to the variability in measurements and the few pilot experiments performed, my results do not conclusively prove a relationship one way or the other, although the increase in activity experiments illustrate that in general H^+ tends to follow the increase in lactate appearance (fig. 23 and 24).

Increasing the stimulation rates to intervals of 50, 10 and 5 seconds resulted in tension losses of 2%, 25% and 40% respectively

Fig. 23.

Experiment in which muscle activity is varied. During the first 150 minutes the stimulation rate was 1 tetanus every 200 seconds. During the period from 150 minutes to 160 minutes the stimulation was increased to 1 tetanus every 5 seconds. Tension changes are shown in the graph at the top. In the bottom graph lactate efflux is indicated by the solid lines and H^+ efflux values are given by the dashed lines.

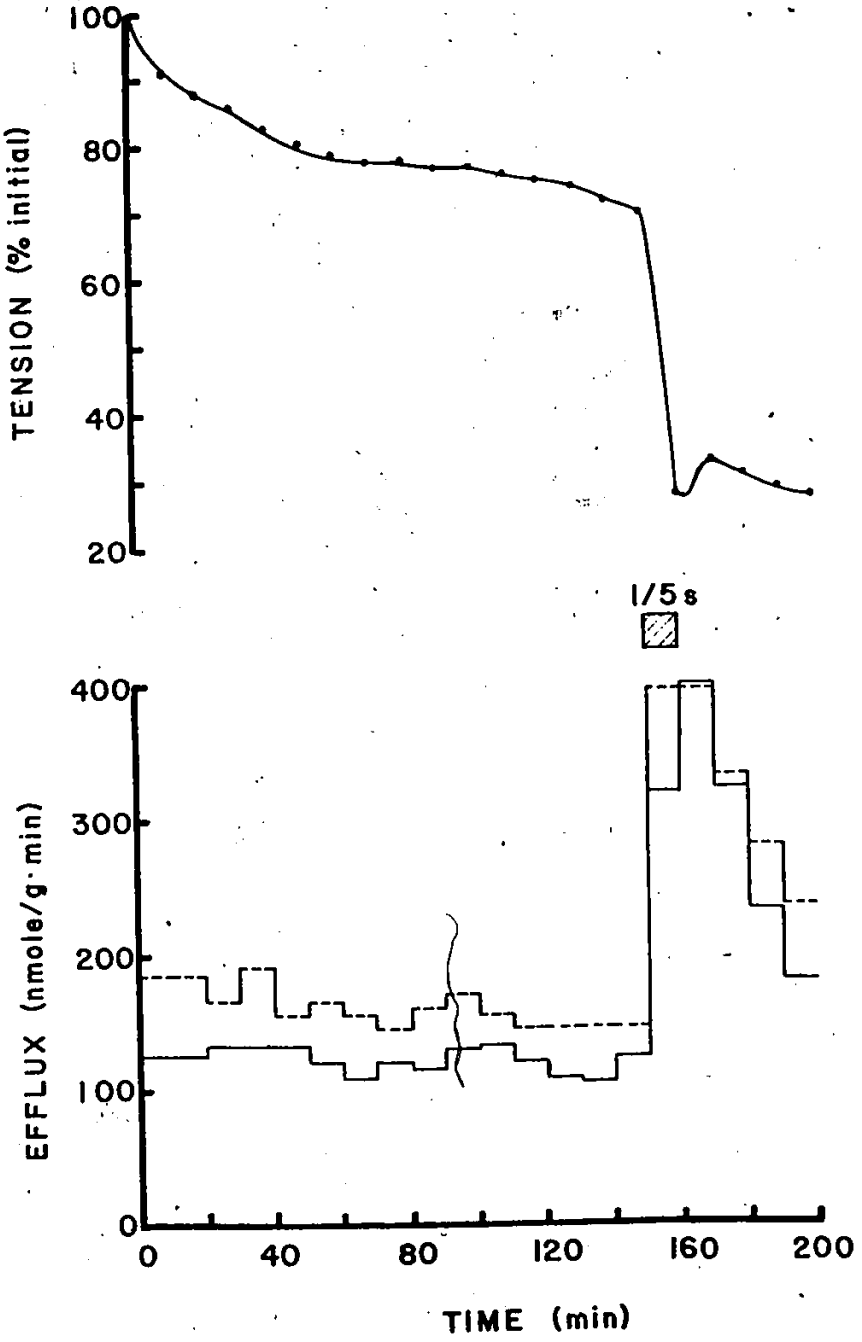
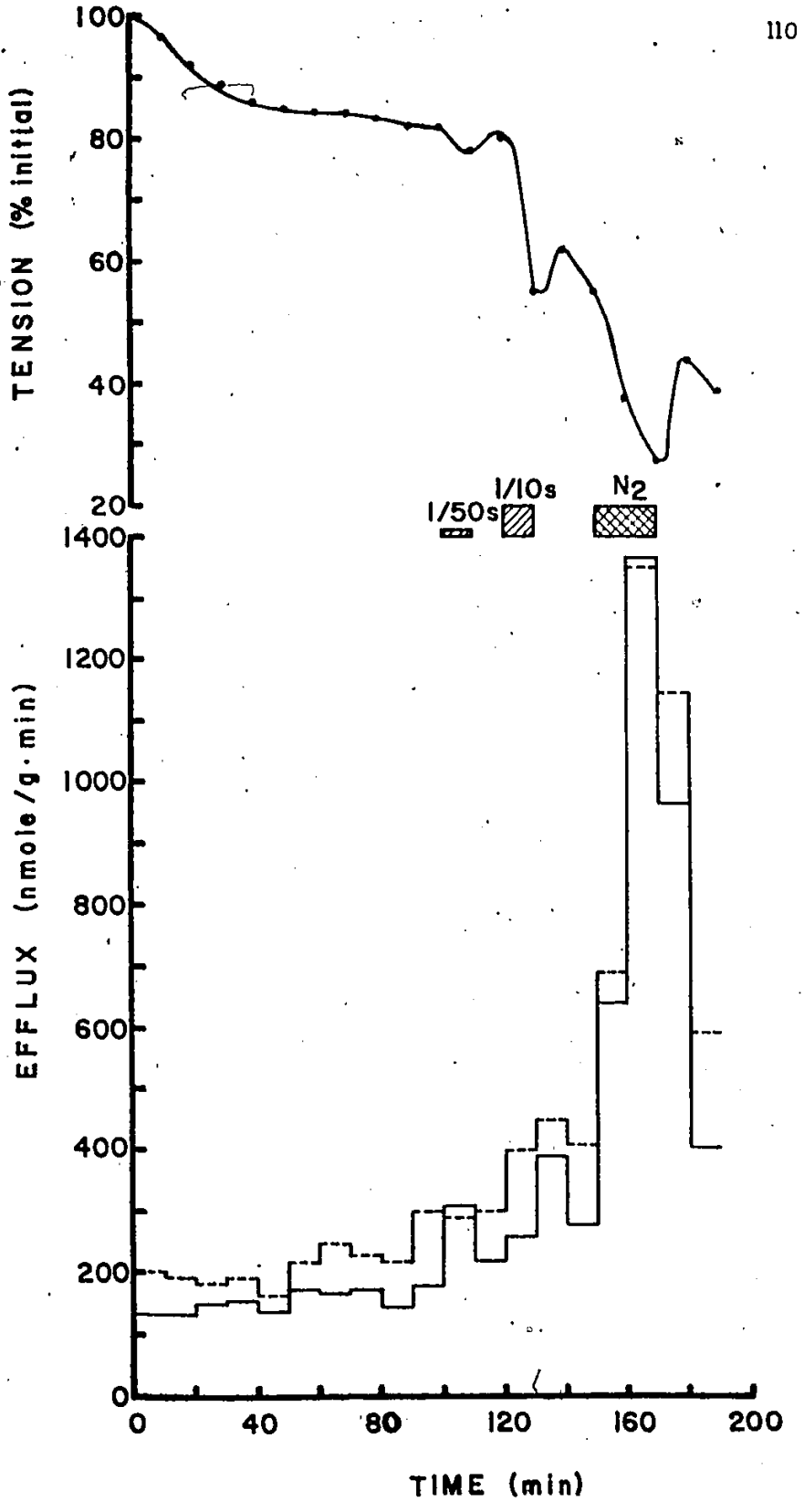

100
80
60
40
20
TENSION (% initial)
1/5 s
400
300
200
100
0
EFFLUX (nmole/g·min)
0
40
80
120
160
200
TIME (min)

Fig. 24.

Effects of increased activity and anoxia on muscle tension lactate efflux and H^+ efflux. Stimulation frequency was increased to 1 tetanus/50s and 1 tetanus/10s for 10 minutes each during the time periods of 100-110 minutes and 120-130 minutes. At all other times the stimulation rate was 1 tetanus/200s. During the period from 150-170 minutes N_2 replaced the O_2 supply to the muscle. The top graph shows the changes in tension. In the bottom graph the lactate efflux is indicated by the solid line and H^+ efflux by the dashed line. Results are for one experiment only.


TENSION (% initial)
100
80
60
40
20
1/50s
1/10s
N2
EFFLUX (nmole/g·min)
1400
1200
1000
800
600
400
200
0
0
40
80
120
160
200
TIME (min)

(fig. 23 and 24).

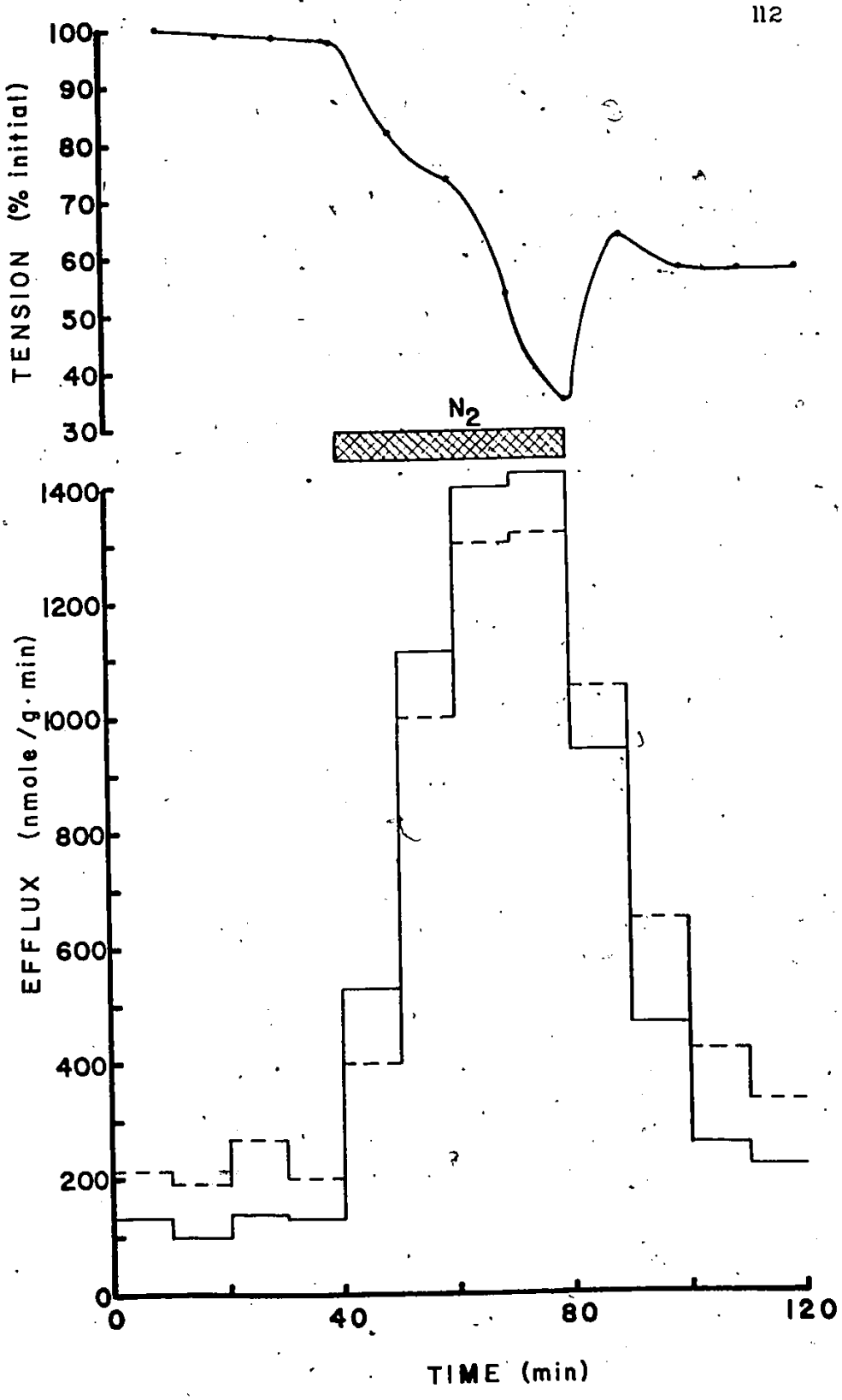
2. Effects of Anoxia on Lactate Efflux, H^+ Efflux and Muscle Tension

As well as increasing activity to exceed a level of energy requirement corresponding to the maximum O_2 consumption, and therefore increasing lactate production, it is also possible to decrease or remove entirely the O_2 supply to the muscle tissue, giving rise to an increase in lactate production. In two experiments the O_2 was completely replaced with N_2 for periods of 20 and 40 minutes. In each of the two experiments, the maximum lactate efflux was about 1400 nM/g per minute (fig. 24 and 25). Restoration of the O_2 supply resulted in the rapid return of lactate efflux towards the resting condition level.

Net acid efflux tended to follow the changes in lactate measurements. However, at the beginning of the anoxic conditions lac-

Fig. 25.

Effects of anoxia on muscle tension, lactate efflux and H^+ efflux. During the time period from 40 minutes to 80 minutes the muscle was equilibrated with a 5% CO_2 and 95% N_2 gas mixture instead of the normal 5% CO_2 and 95% O_2. Upper graph indicates changes in muscle tension. Lower graph is lactate efflux, (solid lines) and H^+ efflux, (dashed lines). Results are for one experiment only.


TENSION (% initial)
100
90
80
70
60
50
40
30
N_2
EFFLUX (nmole/g·min)
1400
1200
1000
800
600
400
200
0
0
40
80
120
TIME (min)

tate efflux tends to increase more rapidly than the H^+ efflux. Upon return to normal oxygenation, the net acid efflux tends to return more slowly to its resting efflux than does lactate (fig. 24 and 25).

Tension is drastically reduced when the muscle is exposed to anoxic conditions. In fig. 25 at the end of 40 minutes of anoxia, tension has been reduced to 35% of the initial value. Resumption of the O_2 supply restores about 60% of the pre-anoxic tension.

CHAPTER IV

Discussion

A. Muscle Tension

In the present experiments, acidosis produced by an increase in pCO_2 has a stimulating effect on the maximal tetanus of the rat diaphragm, while acidosis produced by decreasing the bicarbonate concentration depressed the isometric tension. These results are similar to those of Pannier, Weyne and Lausen (1970) for the isolated soleus muscle of the rat. However, Creese (1950, 1958) reported that increased pCO_2 depressed the twitch contraction of rat diaphragm although lowering the bicarbonate concentration had the same depressive influence I observed. In dealing specifically with this matter, Pannier et al (1970) noted that various types of isolated muscle preparations react in strikingly different manners toward acidosis.

Creese (1950) suggested the effect produced upon muscle tension by changes in bicarbonate concentration and pCO_2 resulted from changes in intracellular pH. The results of my experiments and Pannier et al (1970) do not support this idea since a decrease in pH_i due to increases in pCO_2 or decreases in $[HCO_3^-]$ results in an enhancement or depression respectively of maximum tetanic tension. The only factor which varies positively with the tension changes is that of the intracellular bicarbonate. It has been suggested that bicarbonate has an accelerating effect on certain ionic exchanges across the erythrocyte membrane (Creese, 1950) but this has not been shown for muscles.

B. Lactate Efflux

1. Resting Levels of Lactate Efflux

As indicated in the results, the lactate efflux initially began at a high value of 400 nM/g

per minute and gradually declined at the end of thirty minutes to an average of 150 nM/g per minute. Although the absolute levels are not the same this pattern of initially high values is similar to results obtained for perfused (Hollanders, 1968; Rowlands, 1969) and Bülbring-type (Rowlands, 1969) rat diaphragm preparations. This high initial lactate level is especially pronounced in stun-killed rats as opposed to anaesthetized victims. Both investigators (Hollanders, 1968 and Rowlands, 1969) offered possible solutions to explain this pattern of lactate efflux.

It had been proposed by Hollanders (1968) that this elevated level at the start of perfusion was possibly due to three things: adrenaline release caused by handling and killing the animal, the loss of bound insulin, and hypoxia occurring between killing and the start of perfusion. However, Rowlands (1969) provided evidence that the initial lactate level was not the result of either adrenaline or insulin but was an inevi-

table event in this preparation. She showed that the initial lactate was derived from muscle glycogen and that, after about thirty minutes, substrate for glycolysis comes from glucose in the bathing medium.

Following the washout and time for equilibration, a steady lactate output level remained at an average of 120 nM/g per minute with a range in value of 90-150. This is less than values obtained by Hollanders (1968) and Rowlands (1969 a and b) in perfused and Bülbring-type rat diaphragm preparations. Their results gave control lactate efflux levels of about 330 nM/g per minute. However, the values obtained for lactate production from incubated diaphragms are similar to my own. These results were 104-124 nM/g per minute (Wallaas and Wallaas, 1950) and 80 nM/g per minute (Beatty et al 1960).

The question to be asked is: are low lactate efflux values specifically attributable

only to the incubated diaphragm preparation? In her first paper in 1969, Rowlands explained the low values of lactate efflux from incubated diaphragms as the result of high lactate concentration buildup in the medium to 3.7 mM. However, this does not account for the low values I obtained since, at the end of a ten minute period, medium lactate concentrations were only 0.12 mM which is comparable to the 0.08 mM after 15 minutes of Rowlands' Bülbring preparation. Therefore, the low lactate efflux I observed cannot be explained on the basis of its being measured from an incubated preparation.

The discrepancy in resting lactate production between my results (120 nM/g per minute) and perfused preparations (300 nM/g per minute) may be explained in four possible ways: hypoxia, level of activity, diet of rats, and experimental temperature.

When considering the first possibility, the results of Creese et al (1958) must be taken into account. He showed that diaphragms from

rats 150-250 grams in weight need only 60% O_2 in the stream of gas bubbles flowing past the muscle to maintain fibre potentials up to a depth of 250 μ. My rats weighed from 150-200 grams having an average diaphragm thickness of 490 μ with a range from 410 to 550 μ. Therefore, with strong bubbling on both sides of the muscle in 95% O_2, oxygenation should be more than sufficient to maintain the middle fibres. However, it is doubtful if the higher lactate production results of Rowlands (1969 a) were due to hypoxia since oxygen consumption was higher in her perfused preparations than in the incubated preparation of Kratzing (1952) and Creese et al (1958).

Activity of the muscle may have been a factor maintaining high lactate production in Hollanders' 1968 results which were stimulated for 400 μsec and 40 V at 6/minute. Rowlands also used similar levels of stimulation for the perfused diaphragms although her unstimulated

diaphragms also resulted in the same high levels of lactate production. My results showed that a stimulation rate of 1 tetanus/50 seconds is necessary to raise lactate efflux levels to be comparable to Hollanders (1968) and Rowlands (1969).

Another interesting hypothesis which could possibly explain the low lactate efflux I obtained, is the difference in the previous diet fed my rats compared with those of Hollanders and Rowlands. Hansen et al (1951) showed that rats whose major source of energy was lard, had increased lactate production 2.5 times that of rats fed dextrin for a 3 week period prior to sacrificing. The diet fed to my rats was the standard Purina Rat Chow with 4.3% fat.

A small difference in temperature also existed. The 36 degrees centigrade the present experiments were performed at is one degree lower than those of others. However, the results of Rowlands (1969 b) indicate that the effect

of this temperature difference on lactate efflux would be minimal.

The above four possibilities have been proposed to explain the low lactate production levels I observed compared with the higher values of two other investigators. Exactly which variable or combination of variables is responsible for the difference cannot be specifically stated unless a careful investigation is made comparing the various methods and apparatus. Special emphasis could be placed on the factors of oxygenation, activity and diet and their control of lactate production.

2. Increase in Lactate Efflux During Stimulation and Anoxia

The results in this study have indicated that one supramaximal tetanus (150 cycles/sec., 0.2 sec.) every 50 seconds is sufficient to cause a noticeable increase in lactate efflux. This can be compared with the results of Hollanders'

(1968) in which single shocks at 120/minute for 10 minutes were necessary to produce the first detectable increase.

A comparison can be made between the maximum lactate efflux determined from my experiments and the results of other investigators. Hollanders measured an efflux of lactate as high as 1800 nM/g per minute using single shocks at 3000/minute for 2-3 minutes. The largest efflux rate due to increased activity in my experiments was in response to fatigue developed in the muscle by 1 tetanus/sec for 200 seconds.

This measurement was part of some preliminary experiments which were used to establish the time course of the return of lactate efflux to normal following fatigue. Therefore, it was not determined if this is the maximum rate of efflux in response to increased activity in my system and it is difficult to compare with Hollanders' results. Nonetheless, the results

obtained during anoxia indicate a maximum efflux rate (1400 nM/g per minute) which is comparable to the results of others.

3. Movement of Lactate Across the Membrane

There are two basic possibilities as to how lactic acid crosses the membrane. Either the lactate ion (L^-) is sufficiently permeable to cross the membrane or the lactic acid molecule (HL) is transported. Exactly which possibility is operative is important with respect to acid base balance across the membrane.

a) Lactic Acid Molecule Transport

Much of the original work using kinetic studies assumed the lactic acid molecule moved across the membrane. This was reinforced by results showing a correlation between lactate efflux measurements and net pH changes following increases in the production of lactic acid by the muscle (Henderson 1928; Keul, Doll and Deppler,

1972; Sahlin et al, 1976). This type of mechanism was not supported by Mainwood and Worsley-Brown (1975). During recovery from fatigue in a 10 mM buffer solution, they observed a net hydrogen flux that was only half the net lactate efflux. The results of my experiments have shown a close correlation between net hydrogen and net lactate loss following both increases in activity and subjecting the muscle to anoxia.

However, even if it is the lactate ion which is crossing the membrane, it would be expected that a comparable net hydrogen ion loss would also occur to maintain intracellular pH. If this is the case, then different time courses of the efflux curves during recovery from increased activity or anoxia might be expected. Figures 12, 13, and 14 indicate that this situation possibly exists. My results indicated that the lactate efflux increases as quickly as that of net H^+ efflux. Following return to an oxygenated solution, the rate of decrease in lac-

tate efflux is faster than net H^+ efflux. It must be noted however that the differences are slight and based on only 3 experiments, each with a different protocol. Also, the recent work of Heisler et al (1977) has indicated that there is a higher rate of H^+ efflux.

The equilibration study of Roos (1975) supports passage through the fibre membranes of the undissociated lactic acid molecule. He showed that if it is the undissociated molecules which are passively distributed then the following relationship in the steady state should exist:

$$\frac{[TL]_i}{[TL]_o} = \frac{[H^+]_o}{[H^+]_i}$$

where TL indicates total lactate (L + HL). In his results, Roos obtained a lactate ratio which was 2/3 that of H ions. This disparity could be explained by membrane permeability to the lactate ion. However, when Roos made the same measurements after depolarizing the muscle with potassium there was no change in

this ratio. This work by Roos appears to be the strongest argument for the transfer of HL across the fibre membrane.

More recently, however, some measure of doubt has been placed upon the results of Roos. Hinke and Menard (1976), using barnacle muscle fibres to demonstrate the similarities between pH_i from DMO and microelectrode techniques, showed that there is a more favourable comparison if the value of pH_i - DMO is determined by extrapolating the slow uptake phase of the indicator compounds ^{14}C DMO and ^{3}H inulin to time zero rather than from the "plateau" region. Hinke recalculated Roos' internal pH (DMO) values according to his own regression line determined from pH_i - DMO and pH_i - microelectrode. The result is a poor correlation between lactate and hydrogen ratio rather than a strong correlation.

My results are not consistent with the view that lactate leaves the rat diaphragm

entirely as lactic acid. I have shown that a fall in extracellular pH from 7.5 to 6.9 causes a rapid decrease in lactate efflux. The efflux falls by about 75% and the low efflux rate persists as long as the extracellular pH is low. The effect on lactate efflux is similar whether the pH fall is due to a decrease in HCO_3^- or an increase in CO_2 (figs. 11D and 12).

If lactate efflux is in the form of the undissociated lactic acid molecule, then we must suppose that a fall in external pH either reduces membrane permeability to the acid or that the concentration gradient of the acid across the membrane is decreased.

It seems unlikely that an electrostatic interaction between external protons and ionized groups on the outer surface of the membrane would affect the diffusion of an uncharged molecule through the lipid portion of the membrane which would have to be the case if a change in external pH affected the permeability of the

membrane to lactic acid.

An increase in pCO_2 would be expected to lower internal pH and therefore increase the internal lactic acid level as a fraction of the total internal lactate. This should increase the lactic acid gradient across the membrane since total muscle lactate is not decreased by an increase in pCO_2. This would be expected to increase the rate of lactate efflux.

In addition the intracellular lactate results (table III) indicate that the lactate concentration gradient across the membrane is not significantly altered when the pH_e is decreased by lowering extracellular HCO_3^-.

It therefore appears that the observed effect of pH changes on lactate efflux are not consistent with lactate leaving the cell only in the form of the undissociated acid.

b) Lactate Ion Transport

It has been demonstrated that frog muscle exhibits a definite permeability to the

lactate ion which is about 0.07 that of chloride permeability (Woodbury and Miles, 1973). Their conclusions were based on changes in characteristic resistance and transmembrane potential after the replacement of one anion by another in the solution bathing frog skeletal muscle. Their results are compatible with those of Mainwood and Worsley-Brown (1975) which indicate that in low buffer and pH the efflux of lactate can be 80-90% the result of the transfer of the ion.

It has also been demonstrated that the permeability of chloride-like anions (of which lactate is one) are sensitive to the extracellular pH (Hutter and Warner, 1967 a and b; Woodbury and Miles, 1973). A decrease in external pH will result in a decrease in permeability of the anion. I have already indicated that for the rat diaphragm in these experiments the lactate efflux changes appear to respond primarily to

the external pH and not the ratio of H^+ across the membrane. The decrease in efflux of lactate upon change to low pH_e as a result of decreased HCO_3^- could then be a result of the pH dependence of the anion, indicating that it is primarily the lactate ion which is transferred across the membrane.

c) Other Possible Mechanisms

The most simplistic approach to this problem is to assume that lactate must cross the membrane as either the molecule or anion and that this is controlled mainly by the external pH. If this is the case, then as previously indicated, the most likely candidate is the transfer of the lactate ion. However, the studies of Mainwood and Worsley-Brown gave evidence, for frog sartorius muscle, that it is the molecule which is mainly transferred at high extracellular buffer values and high pH. Roos (1975), also held to a theory proposing the transfer of the molecule to be predominant. He did not attempt

to distinguish between the two possibilities (L^- or HL) at different external buffer and pH values.

Thus, the possibility exists that more light could be shed upon the subject by reproducing experiments similar to those of Mainwood and Worsley-Brown, using the present system. This would include altering the pH with buffers other than bicarbonate and depolarizing the muscle with potassium to separate movements of the lactate ion and lactic acid molecule over a range of pH values. Another approach would be to study the effect of zinc and copper ions on the efflux of lactate at different extracellular buffer concentrations and pH, since these substances are known to block the efflux of chloride-like anions.

Another possible mechanism is that the reduction in lactate efflux may not be due to an effect of pH on the membrane but rather that the reduction in intracellular pH is inhibiting

the glycolytic enzyme phosphofructokinase (Trivedi and Danforth, 1966; Gessen, 1976). Two reasons, however, suggest that this is unlikely.

First, the change in pH_e from 7.5 to 6.9 by both an increase in CO_2 and a decrease in HCO_3^- resulted in an immediate decrease in lactate efflux to 25% of the original efflux. Since the bicarbonate decrease would not be expected to immediately alter the intracellular pH, a quick decrease in lactate efflux is unexpected. Secondly, from measurements made in this laboratory, it can be seen that intracellular lactate does not decrease significantly from the beginning to the end of a 60 minute period in low bicarbonate (Table IV).

C. Acid Base Exchange

1. Steady Net Acid Efflux

As indicated in the results, there is a steady net acid efflux of about 250 nM/g per

minute. There are two possible sources giving rise to the changes in pH from which the net acid efflux is derived. Either this change in pH is intrinsic to the muscle bath system and the method of recording, even in the absence of a muscle strip, or it is a product of the muscle itself.

Controls at the beginning and end of each experiment show that the change in pH is not likely to be a product of leaving the Tyrode in the muscle bath and equilibrating it with different gas mixtures. Also, control experiments without any muscle showed that there is no net change in the pH of a test solution which had spent 10 minutes or more in the muscle bath. A similar volume of Tyrode removed immediately from the reservoir bath had the same pH.

The other possible source of this net acid efflux is the muscle itself. This efflux could be caused by the production of metabolites effluxing across the membrane and/or the loss of intra-

cellular buffers and other substances which might be related to the gradual decline of the cell leading to eventual death.

The direct assay of lactate and pyruvate indicates that their total efflux is approximately 60-70% that of the net acid efflux under resting conditions. Whether this is the result of transfer of the ionic or molecular forms of these substances, the result will be the same since the maintenance of pH_i eventually requires a net acid efflux to balance the transmembrane ratio of H^+.

Consideration was also given to the possibility that damaged fibres were depositing substances such as phosphates and proteins into the bathing medium and thus altering its buffer value. It is unlikely that this is the situation for the following three reasons. First, morphological studies performed in this laboratory revealed the presence of very few damaged fibres (~3%) for this preparation. Secondly, titration

experiments were unable to account for the presence of these substances. Fig. 9 of the results shows a titration in a CO_2 free atmosphere for extracellular fluid collected over a 30 minute period compared with a titration of the original Tyrode. The change in non-bicarbonate buffering ($\Delta\beta_{NB}$) determined from these titrations is less than 0.02 uM/pH unit. The total buffering power (β_T) for the normal Tyrode solution in 5% CO_2 is approximately 1000 uM/pH unit and the ratio $\Delta\beta_{NB}/\beta_T$ is 2×10^{-5}. This excludes the possibility that any buffer or weak acid contributes significantly. Finally, the efflux of phosphate buffers was nullified by the lack of any measured amounts in the bathing solution as is indicated in the results.

The evidence indicates that the net acid efflux under resting conditions is not attributable to the muscle bath nor the method of measurement. However, only 70% can be accounted for by the movement of H^+ (HCO_3^- or OH^-) associated

with the transmembrane efflux of lactate and pyruvate. The possibility exists that the remaining 30% could be accounted by the transfer of smaller amounts of other organic acids.

2. Effects of pCO_2 on Acid Base Exchange

The measured net acid efflux does not speak to the dilemma of what ionic species (HCO_3^-, OH^- or H^+) is actually being transferred. However, for convenience, most of the following discussion will refer only to the net transfer of HCO_3^-.

The observed acid base exchange, from measurements in the medium bathing the muscle, does not tell the whole story. An understanding of the expected changes within the muscle fibres is necessary to predict what forces these fluxes are responding to and to compare my results with those of others. Therefore, I will first examine what changes are expected to occur from a theoretical point of view followed by a

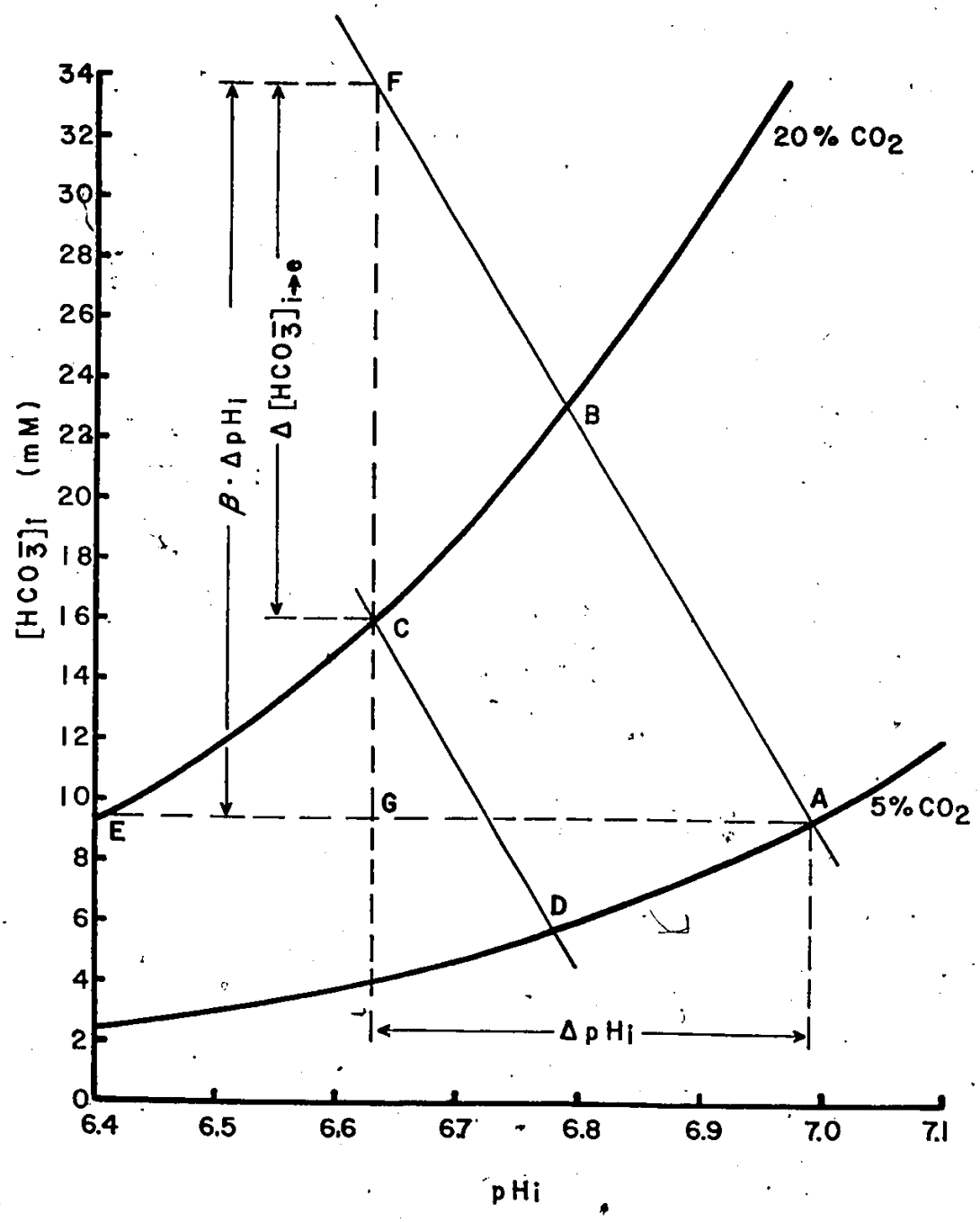
quantitative examination of the results.

Since CO_2 readily diffuses across the membrane and the pCO_2 of the bathing solution is known, then CO_2 isobars can be calculated from the Henderson-Hasselbach equation relating intracellular $[HCO_3^-]$ to the pH_i. This is illustrated by the thick curved lines in fig. 26 for both 5% and 20% CO_2. Another of the controlling features of the intracellular milieu, with respect to pH_i, is the non-bicarbonate buffer value (β). This is represented by the thin unbroken lines with slope $-\beta$ connecting the CO_2 isobars in fig. 26. This buffer value (68 meq/l) is based on estimates of homogenized muscle as opposed to the intact muscle which allows efflux (Heisler and Piiper, 1971).

Let us first consider the theoretical changes in a single muscle fibre. If we assume for simplicity's sake that CO_2 diffusion is very rapid compared with HCO_3^- movement, then a sudden increase in CO_2 (5% to 20%) results in

Fig. 26.

The relationship between H^+, pCO_2 and the non-bicarbonate buffer value (β). Point A represents intracellular conditions for muscles bathed in normal Tyrode based on estimates of pH_i at a given pCO_2 (Heisler, 1975). The change from A→B (β = 68) represents the immediate response to an increase in pCO_2. Final conditions are represented by point C. This requires a net loss of HCO_3^- (F-C).


34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
[HCO3]i (mM)
6.4
6.5
6.6
6.7
6.8
6.9
7.0
7.1
pHi
F
B
C
G
E
A
D
20% CO2
5% CO2
β · ΔpHi
Δ [HCO3]i
ΔpHi

a quick change in pH_i from point A to B (fig. 26). This represents approximately a 14 m M increase in intracellular bicarbonate.

There are three possibilites which can occur from this point. The first is that the system is a closed one and the HCO_3^- concentration and pH_i remain at point B. Secondly, all the increased HCO_3^- may leave the cell until the previous $[HCO_3^-]_i$ level (point E) is reached with the muscle fibre responding as a completely open system, in terms of bicarbonate. The third possibility is that the HCO_3^- responds as an open system but in a limited manner so that intracellular bicarbonate decreases to a value C anywhere between points B and E.

The experiments performed in this study indicated that there is a substantial net base exchange following a change in pCO_2 (fig. 15). The results for the first hour following the change in pCO_2 are plotted against pH_e in fig. 16. The graph is essentially linear over a pH_e range of 6.88

to 7.89.

Shown for comparison are results calculated from the results of Heisler (1975). The values for this line were determined from the difference between the total bicarbonate generated in a closed system (muscle homogenates) and the intracellular bicarbonate values estimated by the DMO method in intact muscle after equilibration at different CO_2 levels while allowing acid base exchange with the bathing medium. There is general agreement between the two graphs with the greatest deviation occurring at the low pCO_2 value.

The net base exchange is indicated by the left-hand side of the following equation:

$$\Delta (HCO_3^-)_{e \rightarrow i} = \beta \cdot \Delta pH_{1,2} + [HCO_3^-]_{i2} - [HCO_3^-]_{i1}$$

where subscripts 1 and 2 represent respectively the conditions before and after a change from $(CO_2)_1$ to $(CO_2)_2$,

β = intracellular non-bicarbonate buffer

and $\beta \cdot \Delta pH_i$ represents the total bicarbonate formed or decomposed due to the non-bicarbonate buffer value.

If we accept Heisler's (1975) values for intracellular pH at different pCO_2's with a constant bicarbonate concentration, then it will be possible to calculate the total bicarbonate formed or decomposed ($\beta \cdot \Delta pH_i$) due to the non-bicarbonate buffer values. (fig. 26; F G). These values (column f, table IV) can be compared with the actual measured efflux of HCO_3^- (column g, table IV). Although there will be some error directly comparing one set of experimental results with another, it can be seen that most of the bicarbonate formed in response to increased CO_2 leaves the muscle cells within one hour following the increase. When the pCO_2 was decreased (5% to 2% CO_2), the predicted loss of intracellular bicarbonate was even less than the amount which entered the

TABLE IV Comparison of measured and predicted base exchange values

a	b	c	d	e	f	g	h
Experiment	pH_e (init.)	pH_e (final)	pH_i (init.)	pH_i (final)	$\beta \cdot \Delta pH_i$ mM/kg	$\Delta(HCO_3^-)_{i \to e}$ M mM/kg	$\Delta(HCO_3^-)_{i \to e}$ NM mM/kg
2% CO_2	7.47	7.89	6.99	7.22	-9.43	-12.79	-5.67
10% CO_2	7.47	7.19	6.99	6.88	4.51	3.93	3.36
20% CO_2	7.47	6.88	6.99	6.63	14.76	14.02	8.53

pH_e initial and final were measured directly.

pH_i initial and final were predicted from Heisler (1975) for similar conditions.

$\beta \cdot \Delta pH_i = (HCO_3^-)_{total}$. β is determined from Heisler & Piiper (1972) non-bicarbonate buffer measured in muscle homogenates.

$\Delta(HCO_3^-)_{i \to e}$ M assumes movement of metabolic acids in dissociated form.

$\Delta(HCO_3^-)_{i \to e}$ NM assumes undissociated movement of metabolic acids; net base transfer corrected for that which would be attributable to metabolic acid movement.

muscle fibres.

However, these calculations (column g) were made on the assumption that lactate and pyruvate effluxed from the muscle fibres in the anionic form, independent of the acid base exchange. If we were to assume that the acid molecule best represented the mode of efflux and subtracted from the net acid efflux H^+ comparable to the measured lactate + pyruvate values, then the measured bicarbonate movements would be somewhat different. Column h of table IV illustrates the results obtained from the non-metabolic net acid efflux. Under these circumstances approximately 64% of the bicarbonate formed (or decomposed), enters (or leaves) the cell during the first hour following the change to a different pCO_2.

Therefore, the rat diaphragm acts as an open system, with respect to bicarbonate, after a change in pCO_2. However, using the pH_i results of others coupled with my measured acid

base exchange, it would seem that the intracellular bicarbonate does not return to the original level prior to the pCO_2 change. Further evidence for an open system is obtained from my results in which the extracellular concentration was decreased from 28mM to 6.6 mM. In this case, the intracellular bicarbonate and pH_i in fig. 26 both decrease while following the path of the 5% CO_2 isobar from point A.

3. Permeability of Bicarbonate

Equilibrium studies have shown that there is an acid base exchange following exposure to a different pCO_2 which accounts for more than half of the intracellular bicarbonate formed or decomposed (Heisler and Piiper, 1971; Heisler, 1975). The present experiments which measured net acid efflux in the first hour following a pCO_2 change have also given similar results. The question remains: is it hydrogen ions or bicarbonate ions that are being transferred?

Mainwood (1966), in his membrane potential measurements of sucrose-washed frog muscle, has shown that it is most likely bicarbonate ions which cross the membrane. He obtained a fall in membrane potential in response to increased pCO_2. However, a fall in pH in the presence of phosphate buffer did not produce a decrease in membrane potential pointing to the bicarbonate ion as the most likely source of the current. Williams et al (1971) also showed that the measurement of H^+ permeability by Woodbury (1970) was insufficient to account for their CO_2-induced depolarization of rat muscle.

The original suggestion was made by Boyle and Conway (1940) that HCO_3^- moves out of the muscle in response to an increase in the intracellular bicarbonate following an increase in pCO_2. This was also implied in a paper by Harris (1963). However, a permeability coefficient of HCO_3^- ($P_{HCO_3^-}$) has not been directly measured.

Nevertheless, a rough estimate of the ratio of bicarbonate permeability to chloride permeability ($P_{HCO_3^-}/P_{Cl^-}$) has been determined. Using excised frog sartorius muscle and measuring transmembrane voltage and resistance after a change in HCO_3^- concentration of the bathing medium, Woodbury (1970) estimated the ratio $P_{HCO_3^-}/P_{Cl^-}$ to be about 0.1. Woodbury and Miles (1973) also estimated the conductance of bicarbonate to be about 0.05 the conductance of chloride in frog muscle based on changes in resistance and transmembrane potential after the replacement of one anion by another in the bathing solution.

To make these ratios meaningful it is necessary to know the values of chloride permeability. These have been determined by two methods. First, from the flux of chloride and secondly from conductance measurements. Measurements of chloride flux have given values for the chloride permeability coefficient of 0.6→1.5 x 10^{-6} cm/sec for frog sartorii (Adrian 1958, 1961). Conductance

measurements have given P_{Cl^-} values of 1.7→ 2.9 x 10^{-6} cm/sec in frog sartorius fibres (Adrian and Freygang 1962; Hubbard, 1963).

Using the permeability ratio ($P_{HCO_3^-}/P_{Cl^-}$) of 0.1 determined by Woodbury (1970) we have a possible range for permeability coefficient of bicarbonate from 0.6 x 10^{-7} cm/sec to 2.9 x 10^{-7} cm/sec. However, if the ratio of HCO_3^- conductance to chloride conductance $G_{HCO_3^-}/G_{Cl^-}$ = 0.05 determined by Woodbury and Miles is more accurate the range of values is then 0.3 x 10^{-7} cm/sec to 1.45 x 10^{-7} cm/sec. Combining the two possible extremes for the HCO_3^- permeability coefficient, we obtain values as low as 0.3 x 10^{-7} to a high of 2.9 x 10^{-7} cm/sec.

From the results of my experiments it was possible to estimate an apparent permeability coefficient for bicarbonate. It is necessary to assume that the change in net efflux (ΔJ) immediately after a change in pCO_2 or $[HCO_3^-]$ is due to a bicarbonate efflux in response to a

change in the electrochemical potential of bicarbonate across the cell membrane. This change in net HCO_3^- efflux (ΔJ) can be used to describe bicarbonate permeability in terms of the constant field equation:

$$J_A = P_A \frac{VF}{RT} \left(\frac{A_e - A_i \exp^{-\frac{VF}{RT}}}{1 - \exp^{-\frac{VF}{RT}}} \right) \quad (1)$$

J_A = anion flux, inward flux being positive

P_A = anion permeability

A_e = extracellular anion concentration

A_i = intracellular anion concentration

From equation (1), the change in flux (ΔJ_A) due to a change in CO_2 is:

$$\Delta J_{A_{1\to2}} = P_A \cdot \frac{VF}{RT} (A_{i2} - A_{i1}) \frac{\exp^{-\frac{VF}{RT}}}{1 - \exp^{-\frac{VF}{RT}}}$$

where subscripts (1) and (2) represent

respectively the conditions before and after a change from $(CO_2)_1$ to $(CO_2)_2$.

Thus:

$$P_A = \Delta J_A \cdot \frac{1 - \exp^{-\frac{VF}{RT}}}{\exp^{-\frac{VF}{RT}} \cdot \frac{VF}{RT}} \cdot \frac{1}{(A_{i2} - A_{i1})}$$

and after a change in HCO_3 from A_{e1} to A_{e2}:

$$P_A = \Delta J_A \cdot \frac{1 - \exp^{-\frac{VF}{RT}}}{\frac{VF}{RT}} \cdot \frac{1}{(A_{e1} - A_{e2})}$$

It is important that the membrane potential value does not change significantly. For the calculations presented in Table V the membrane potential was assumed to be -70mV. A range in membrane potential from -60 to -80 mV results in less than a 10% change in the bicarbonate permeability coefficient.

Not only will the value for the membrane potential affect the calculated $P_{HCO_3^-}$ but

TABLE V: Net acid base flux in the rat diaphragm and estimates of the apparent $P_{HCO_3^-}$

Experiments	n	ΔJ (nmole/g · min) uncorr.	corr.*	$P_{HCO_3^-}$ ** (cm/sec)* uncorr.	corr.
CO_2 5→2%	4	213	95	2.62×10^{-7}	1.17×10^{-7}
CO_2 5→10%	4	66	55	0.94×10^{-7}	0.80×10^{-7}
CO_2 5→20%	4	341	266	1.86×10^{-7}	1.45×10^{-7}
HCO_3^- 28→6.6mM	4	461	387	2.15×10^{-6}	1.81×10^{-6}

*Corrected values for ΔJ are obtained by subtracting the change in net efflux of lactate + pyruvate occurring simultaneously with the change in CO_2 or HCO_3^-.

** A value of 932 cm^2/g used to calculate flux per unit membrane area, determined by morphometric methods.

consideration must also be given to the active transport of H^+ (HCO_3^- or OH^-) and the form in which the metabolic acids lactate and pyruvate are transferred across the membrane (molecular or ionic). Since the change in efflux of lactate and pyruvate was measured it is possible to determine the correction factor to the ΔJ value if we were to assume that these acids were in the undissociated form. This correction factor substantially changes the $P_{HCO_3^-}$ for the CO_2 change from 5% to 2%, but has relatively little effect on the other values (Table V). It is possible also to obtain some idea of the effect of the H^+ (HCO_3^- or OH^-) pump on the calculated $P_{HCO_3^-}$ by comparing the results obtained for an increase in CO_2 (5% to 20%) and a decrease in $[HCO_3^-]_e$ (28 mM to 6.6 mM). Inhibition of the pump due to a fall in $[HCO_3^-]$ and pH_e resulted in errors leading to high values of ΔJ and $P_{HCO_3^-}$. The estimates obtained by the two types of experiments vary by a factor

of 10 (Table III) indicating that the active transport has a substantial influence.

Table V indicates a range in estimated P_{HCO_3} values for the CO_2 change experiments from 0.80×10^{-7} cm/sec to 2.62×10^{-7} cm/sec. This is within the minimum (0.3×10^{-7} cm/sec) and maximum (2.9×10^{-7} cm/sec) values previously determined from the literature. However, the errors introduced by the inhibition of the active pump have resulted in $P_{HCO_3^-}$ values slightly higher than the maximum determined from the results of others.

The evidence strongly suggests then, that HCO_3^- does cross the membrane following a change in pCO_2 and that this accounts for much of the HCO_3^- formed (or decomposed) in response to this change.

Given that the $[HCO_3^-]_i$ is above the passive equilibrium level and the fact that bicarbonate ions can penetrate the cell membrane there is therefore, an increased workload presented to the H^+ pump or any other mechanism which exists

to maintain intracellular pH. However, a high $P_{HCO_3^-}$ would be of value in the situation in which the internal buffer reserves are depleted. At this time, the HCO_3^- flux can be reversed and help maintain the intracellular pH. An example of this would be during a sudden increase in glycolysis. Assuming the condition that the transmembrane movement of lactate is in the ionic form, the fall in pH_i due to the increased production of this acid would be controlled by the amount of total internal buffer present, changes in the active pump mechanism and the rate of transmembrane influx from outside the cell.

SUMMARY

1. A system was developed to measure tension, efflux of lactate and pyruvate and acid base exchange in rat diaphragm strips. Changes in values for these variables were made in response to anoxia, increases in muscle activity, and changes in the CO_2 level and bicarbonate concentration.

2. An increase in pCO_2 has a stimulating effect on the maximal tetanus tension while a decrease in bicarbonate concentration depressed isometric tension. This suggests that intracellular bicarbonate might play a role in the maintenance of tetanic tension.

3. There is a steady lactate efflux of about 120 nM/g per minute during control conditions. Pyruvate efflux is about 10% that of the lactate efflux. A maximum lactate efflux of 1400 nM/g per minute was observed during anoxia. Lactate efflux was found to be pH dependent and increased from 10 nM/g per minute to 230 nM/g per minute for an extracellular pH increase from 6.2 to 7.9. The results have indicated that lactate is trans-

ferred across the membrane primarily in the ionic form.

4. There is a steady net acid efflux in the order of 250 nM/g per minute during control conditions. The net acid efflux increases following a decrease in pCO_2 and decreases or reverses in response to an increase in pCO_2. A decrease in bicarbonate concentration has an effect similar to that of an increase in pCO_2.

5. Assuming that the changes in acid base exchange in response to changes in pCO_2 are due primarily to passive HCO_3^- movements, then it can be shown that a large fraction of the HCO_3^- generated within the muscle fibres by non-bicarbonate buffers is transferred within one hour following a change in pCO_2. It is possible to calculate an apparent permeability coefficient for bicarbonate which varies under different conditions from a value of 0.8×10^{-7} cm/sec to 2.62×10^{-7} cm/sec.

APPENDIX

Calculation of the Quantity of Acid Added to a Two Buffer System Determined From the Measurement of pH

1. Non-Bicarbonate Buffer

The total amount of buffer (A) is fixed. At any pH an amount aA is in the alkaline form and the amount (1-a)A is in the acid form.

$$pH_1 = pK_A + \log \frac{a_1 A}{(1-a_1)A}$$

$$\frac{a_1}{1-a_1} = 10^{pH_1 - pK_A}$$

$$a_1 = \frac{10^{pH_1 - pK_A}}{1 + 10^{pH_1 - pK_A}} \qquad (1)$$

If x amount of acid is added:

$$pH_2 = pK_A \log \frac{a_2 A}{(1 - a_2)A}$$

$$a_2 = \frac{10^{pH_2 - pK_A}}{1 + 10^{pH_2 - pK_A}} \qquad (2)$$

$$x = -A(a_1 - a_2)$$

$$x = A(a_2 - a_1)$$

Substituting for a_1 and a_2 from equations (1) and (2),

$$x = A\left[\frac{10^{pH_2 - pK_A}}{1 + 10^{pH_2 - pK_A}} - \frac{10^{pH_1 - pK_A}}{1 + 10^{pH_1 - pK_A}}\right] \qquad (3)$$

2. Bicarbonate Buffer

This is an open system buffer. Therefore, the amount of buffer in the acid form (BH) is constant which is determined by the pCO_2. At any pH an amount B is in the alkaline form.

BH can be determined exactly since:

$$pH_1 = pK_B^* + \log \frac{B_1}{BH}$$

$$\frac{B_1}{BH} = 10^{pH_1 - pK_B}$$

*pK_B determined from Andersson (1962)

$$BH = \frac{B_1}{10^{pH_1} - pK_B} \qquad (4)$$

True value of B_1:

If the initial amount of bicarbonate added is B_1^1 then the true value of B_1 is $B_1^1 - y$ where y is the bicarbonate lost due to interaction with other buffers in solution.

Suppose A is added as H_2PO_4

then at pH_1 : $pH_1 = pK_A + \log \frac{y}{A - y}$

where y is the amount of H_2PO_4 converted to HPO_4 which must be equal to the bicarbonate lost.

Now $\frac{y}{A - y} = 10^{pH_1 - pK_A}$

$y = (A-y)\ 10^{pH_1 - pK_A}$

$$y = A \cdot \frac{10^{pH_1 - pK_A}}{1 + 10^{pH_1 - pK_A}} \qquad (5)$$

Since $B_1 = B_1^1 - y$

Substitute for y from equation (5)

$$B_1 = B_1^1 - A \cdot \frac{10^{pH_1 - pK_A}}{1 + 10^{pH_1 - pK_A}} \qquad (6)$$

Substituting B_1 from equation (6) into equation (4) a more precise BH^1 is determined as:

$$BH^1 = \left[B_1^1 - A \cdot \frac{10^{pH_1 - pK_A}}{1 + 10^{pH_1 - pK_A}} \right] \cdot \frac{1}{10^{pH_1 - pK_B}} \qquad (7)$$

and

$$pH_1 = pK_B + \log \frac{B_1}{BH^1}$$

$$B_1 = BH^1 \cdot 10^{pH_1 - pK_B} \qquad (8)$$

If x amount of acid is added:

$$pH_2 = pK_B + \log \frac{B_2}{BH^1}$$

$$B_2 = BH^1 \cdot 10^{pH_2 - pK_B} \qquad (9)$$

However, $x = -(B_1 - B_2)$

$$x = B_2 - B_1$$

Substitute for B_1 and B_2 from equations (8) and (9)

$$x = BH^1 \cdot 10^{pH_2 - pK_B} - BH^1 \cdot 10^{pH_1 - pK_B}$$

$$x = BH^1 \cdot \left(10^{pH_2 - pK_B} - 10^{pH_1 - pK_B}\right) \qquad (10)$$

where BH^1 is first determined using equation (7) from the initial bicarbonate added and the pH of the solution before the addition of any acid from the muscle.

3. Complete System

If a quantity of acid is added to the system then the total amount of hydrogen ions (H_T) added to the Tyrode solution can be determined from the following relationship:

$$H_T = \Delta H^+ - \Delta OH^- - \Delta HCO_3^- + \Delta H_2PO_4^- \qquad (11)$$

Let V be the volume of solution to which x is added.

Then $\Delta H^+ = V \cdot (10^{-pH_2} - 10^{-pH_1})$ (12)

$$-\Delta OH^- = V \cdot (10^{-(14-pH_2)} - 10^{-(14-pH_1)}) \quad (13)$$

from equation (10)

$$-\Delta HCO_3^- = V \cdot BH \cdot (10^{pH_2-pK_B} - 10^{pH_1-pK_B}) \quad (14)$$

from equation (3)

$$\Delta H_2PO_4^- = V \cdot A \cdot \left[\frac{10^{pH_2-pK_A}}{1 + 10^{pH_2-pK_A}} - \frac{10^{pH_1-pK_A}}{1 + 10^{pH_1-pK_A}} \right] \quad (15)$$

If BH and A are in μmoles/ml and the volume is in ml, the answer is in μmoles for $-\Delta HCO_3^-$ and ΔH_2PO_4 and mmoles for ΔH^+ and $-\Delta OH^-$. To get the answer in nmoles, ΔH^+ and $-\Delta OH^-$ should be multiplied by 10^6 and $-\Delta HCO_3^-$ and $\Delta H_2PO_4^-$ should be multiplied by 10^3.

Substituting equations (12), (13), (14)

and (15) into equation (11) with the appropriate changes to obtain the same units then the final form of the equation is:

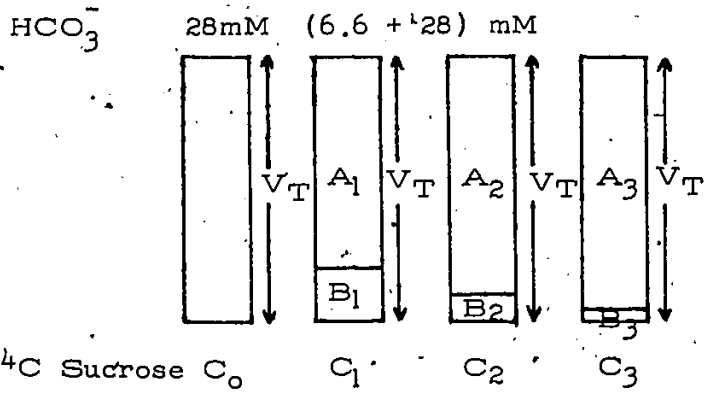
$$H_T = \left[10^6 \cdot (10^{-pH_2} - 10^{-pH_1}) + 10^6 \cdot (10^{-(14-pH_2)} - 10^{-(14-pH_1)}) + 10^3 \cdot BH \cdot (10^{pH_2-pK_B} - 10^{pH_1-pK_B}) + 10^3 \cdot A \cdot \left(\frac{10^{pH_2-pK_A}}{1 + 10^{pH_2-pK_A}} - \frac{10^{pH_1-pK_A}}{1 + 10^{pH_1-pK_A}} \right) \right] \cdot V \quad (16)$$

4. Residual Volume and Changes in HCO_3^- Concentration

When changing from one bicarbonate concentration to another it is necessary to know to what extent the residual amounts of the previous solution affects the concentration of the new solution. When this is known then it is possible to calculate a correction factor to be applied to

the calculation of the amount of acid added in order to determine the contribution by the muscle.

For example, when changing from normal bicarbonate (28 mM) to low bicarbonate (6.6 mM) the effect of residual amounts of first solution can be calculated.



A_1, A_2, ... volume of freshly added 6.6 mM HCO_3^- solution

B_1, B_2, ... residual volumes of previous 28 mM HCO_3^- solution remaining.

C_o = activity (CPM) of ^{14}C sucrose in 28 mM HCO_3^- solution

C_1, C_2, ... activity of ^{14}C sucrose in successive sampling periods.

V_T= total volume in muscle bath

$$B_1 \times C_o = V_T \times C_1$$

$$B_2 \times C_o = V_T \times C_2$$

etc.

and

$$\frac{B_1}{V_T} = \frac{C_1}{C_o} = \alpha_1 \quad \text{and } B_1 = V_T \alpha_1 \text{ also } A_1 = V_T - V_T \alpha_1$$

$$\frac{B_2}{V_T} = \frac{C_2}{C_o} = \alpha_2 \text{ and } B_2 = V_T \alpha_2 \text{ also } A_2 = V_T - V_T \alpha_2$$

$$[HCO_3^-]_1 = \frac{6.6\ A_1 + 28\ B_1}{V_T}$$

Substituting for A_1 and B_1:

$$[HCO_3^-]_1 = \frac{6.6\ V_T (1-\alpha_1) + 28\ V_T \alpha_1}{V_T}$$

$$[HCO_3^-]_1 = 6.6\ (1-\alpha_1) + 28\ \alpha_1 \qquad (17)$$

Therefore the increase in bicarbonate concentration above the 6.6 mM of the freshly added solution is:

$$\Delta [HCO_3^-]_1 = [HCO_3^-]_1 - 6.6 \qquad (18)$$

If equation (16) is used to calculate the total amount of hydrogen ions (H_T) added to the buffer system, then during successive samplings following a change to 6.6 mM HCO_3^- from 28 mM HCO_3^- the muscle component (H_m) is calculated as:

$$H_m = \Delta[HCO_3^-]_1 - H_T$$

$\Delta [HCO_3^-]$ being derived from equations (17) and (18).

A similar line of reasoning can be used to determine the change in bicarbonate concentration due to residual volumes of the previous solution when returning to 28 mM HCO_3^- from 6.6 mM.

REFERENCES

Adler, S. 1972. The simultaneous determination of muscle cell pH using a weak acid and a weak base. J. Clin. Invest. 51: 256-265.

Adler, S. 1972. The interrelationship of metabolism and pH heterogeneity in muscle cells. J. Lab. Clin. Med. 80:351-363.

Adler, S., B. Anderson and B. Zett. 1975. Effect of osmolarity on intracellular pH of rat diaphragm muscle. Am. J. Physiol. 228:725-729.

Adler, S., A. Roy and A.S. Relman. 1965. Intracellular acid-base regulation. I. The response of muscle cells to changes in CO_2 tension or extracellular bicarbonate concentration. J. Clin. Invest. 44:8-20.

Adler, S., A. Roy and A.S. Relman. 1965. Intracellular acid base regulation. II. The interaction between CO_2 tension and extracellular bicarbonate in the determination of muscle cell pH. J. Clin. Invest. 44:21-30.

Ahlborg, B., J. Bergstrom, L.G. Ekelund, G. Guornieri, R.C. Harris, E. Hultman and L.O. Nordesjo. 1972. Muscle metabolism during isometric exercise performed at constant force. J. Appl. Physiol. 33:224-228.

Bass, A., E. Gutmann and M. Hanikova. 1971. Contraction properties and enzyme pattern of the mouse and rat diaphragm. Physiol. Bohemoslov. 20:5-10.

Bate Smith, E.C. 1938. The buffering of muscle in rigor; protein, phosphate and carnosine. J. Physiol. (London) 92:336-343.

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

Benade, A.J.S. and N. Heisler. 1978. Comparison of efflux rates of hydrogen and lactate ions from isolated muscles in vitro. Respiration Physiology 32:369-380.

Bock, A.V., D.B. Dill, L.M. Hurxthal, J.S. Lawrence, T.C. Coolidge, M.E. Dailey and L.J. Henderson. 1927. Blood as a physio-chemical system. V. The composition and respiratory exchanges of normal human blood during work. J. Biol. Chem. 73:749-766.

Boyle, P.J. and E.J. Conway. 1941. Potassium accumulation in muscle and associated changes. J. Physiol. 100:1-63.

Brown, E.B. Jr. and B. Goott. 1963. Intracellular hydrogen ion changes and potassium movements. Amer. J. Physiol. 204:765-770.

Brown, E.B. Jr., Gyum Kim Woo and F.A. Moorhead, Jr. 1967. Intracellular pH during metabolic acidosis of intracellular and extracellular origin. Proc. Soc. Exp. Biol. Med. 126:595-599.

Brownlee, G. and D.W. Straughan. 1957. Motor nerve stimulation and acetylcholine release in the perfused rat phrenic nerve diaphragm preparation. J. Physiol. (London) 136:68.

Bülbring, E. 1946. Observations on the isolated phrenic nerve diaphragm preparation of the rat. Brit. J. Pharmacol. 1:38-61.

Burgen, A.S.V., F. Dickers and L.J. Zatman. 1949. The action of botulism toxin on the neuro-muscular junction. J. Physiol. 109: 10-24.

Burnell, J.M. 1968. In vivo response of muscle to changes in CO_2 tension or extracellular bicarbonate. Amer. J. Physiol. 215:1376-1383.

Caldwell, P.C. 1954. An investigation of the intracellular pH of crab muscle fibres by means of micro-glass and micro-tungsten electrodes. J. Physiol. 126:169-180.

Caldwell, P.C. 1956. Intracellular pH. As quoted in "International Review of Cytology", G.H. Bourne and J.F. Danielli, ed. New York: Academic Press.

Caldwell, P.C. 1958. Studies on the internal pH of large muscle and nerve fibre. J. Physiol. 142:22-62.

Carter, N.W., F.C. Rector Jr., D.S. Campion and D.W. Seldin. 1967. Measurement of intracellular pH with glass microelectrodes. Federation Proc. 26:1322-1326.

Carter, N.W., F.C. Rector, D.S. Campion and D.W. Seldin. 1967. Measurement of intracellular pH of skeletal muscle with pH-sensitive glass microelectrodes. J. Clin. Invest. 46:920-933.

Chambers, R., H. Pollack and S. Hiller. 1927. The protoplasmic pH of living cells. Proc. Soc. Exp. Biol. Med. 24:760-761.

Clancy, R.L. and E.B. Brown, Jr. 1966. In vivo CO_2 buffer curves of skeletal and cardiac muscle. Amer. J. Physiol. 211:1309-1312.

Conway, E.J. and P.J. Fearon. 1944. The acid-labile CO_2 in mammalian muscle and the pH of the muscle fibre. J. Physiol. 103:274-289.

Creese, R. 1950. Bicarbonate ion and striated muscle. J. Physiol. 100:450-457.

Creese, R., N.W. Scholes and W.J. Whalen. 1958. Resting potentials of diaphragm muscle after prolonged anoxia. J. Physiol. 140:301-317.

Danforth, W.H. 1965. Activation of glycolytic pathway in muscle. As quoted in "Control of Energy Metabolism", B. Chance and R.W. Estabrook, ed. New York: Academic Press.

Diamant, B., J. Karlsson and B. Saltin. 1968. Muscle tissue lactate after maximal exercise in man. Acta Physiol. Scand. 72:383-384.

Donnan, F.G. 1911. Theorie der Membrangleichgewichte und Membranpotentiale bei Vorhandensein von nicht dialysierenden Elektrolyten. Ein Beitrag zur physikalisch-chemischen Physiologie. Z. Electrochem. 17:572-573.

Dubuisson, M. 1939. Studies on the chemical processes which occur in muscle before, during and after contraction. J. Physiol. 94:461-482.

Eckel, R.E., A.W. Botschner and D. H. Wood. 1959. The pH of K-deficient muscle. Am. J. Physiol. 196:811-818.

Edwards, R.H.T., R.C. Harris, E. Hultman and L.-O. Nordesjo. 1972. Phosphagen utilization and resynthesis in successive isometric contractions, sustained to fatigue, of the quadriceps muscle in man. J. Physiol. 224:40.

Edsall, J.T. and J. Wyman. 1958. Carbon dioxide and carbonic acid. As quoted in "Biophysical Chemistry". New York: Academic Press.

Eggleton, G.P., P. Eggleton and A.V. Hill. 1928. The coefficient of diffusion of lactic acid through muscle. Proc. Roy. Soc. Ser. B. CIII:620-628.

Fenn, W.O. 1928. The carbon dioxide dissociation curve of nerve and muscle. Am. J. Physiol. 85:207-223.

Fenn, W.O. 1936. Electrolytes in muscle. Physiol. Rev. 16:450-487.

Fenn, W.O. 1961. Carbon dioxide and intracellular homeostasis. Ann. N.Y. Acad. Sci. 92:547-558.

Fenn, W.O. and F.W. Maurer. 1935. The pH of muscle. Protoplasma 24:337.

Fletcher, W.M. 1913-1914. Lactic acid formation, survival respiration and rigor mortis in mammalian muscle. J. Physiol. Lond. 47:361-380.

Fletcher, W.M. and F.G. Hopkins. 1907. Lactic acid in amphibian muscle. J. Physiol. 35:247-307.

Furusawa, K. and P.M.T. Kerridge. 1927. The hydrogen ion concentration of the muscles of the cat. J. Physiol. 63:33-41.

Gesser, H. 1976. Significance of the extracellular bicarbonate buffer system to anaerobic glycolysis in hypoxic muscle. Acta Physiol. Scand. 98:110-115.

Gonzalez, N.C., M. Gerbrant and E.B. Brown. 1976. Changes in skeletal muscle cell pH during graded changes in pCO_2. Respir. Physiol. 26:207-212.

Gonzalez, R.C. and R.L. Clancy. 1975. Inotropic and intracellular acid-base changes during metabolic acidosis. Am. J. Physiol. 228:1060-1064.

Hansen, R.G., W.J. Rutter and L.T. Samuels. 1951. The effect of previous diet on the metabolism of glucose by rat diaphragm.

J. Biol. Chem. 192:243-249.

Harken, A.H. 1975. The influence of pH on lactate metabolism and oxygen uptake in canine skeletal muscle. Am. J. Cardiol. 35:143.

Haugaard, N., J.B. Marsh and W.C. Stadie. 1951. Phosphate metabolism of the isolated rat diaphragm. J. Biol. Chem. 189:59-63.

Heisler, N. 1975. Intracellular pH of isolated rat diaphragm muscle with metabolic and respiratory changes of extracellular pH. Respir. Physiol. 23:243-255.

Heisler, N., P. Nissen and J. Piiper. 1972. Behaviour of intracellular pH in excised rat diaphragms with metabolic changes of extracellular pH. Pflugers Arch. 332: Suppl. R45.

Heisler, N. and J. Piiper. 1971. The buffer value of rat diaphragm muscle tissued determined by pCO_2 equilibration of homogenates. Respir. Physiol. 12:169-178.

Heisler, N. and J. Piiper. 1972. Determination of intracellular buffering properties in rat diaphragm muscle. Am. J. Physiol. 222: 747-753.

Heisler, N. and J. Piiper. 1972. Intracellular pH in excised rat diaphragms with respiratory and metabolic changes of extracellular pH. Pflugers Arch. 335:Suppl. R89.

Heisler, N., H. Weitz and K.F. Rother. 1977. Efflux kinetics of hydrogen and lactate ions from isolated rat diaphragm. Proc. XXVII Int. Cong. Physiol. Sc. 13, 315 Abst. 923.

Henderson, L.J. 1928. Blood. A Study in General Physiology. New Haven: Yale University Press.

Hill, A.V. 1955. The influence of the external medium on the internal pH of muscle. Proc. Roy. Soc. B 144:1-22.

Hill, A.V. and P.S. Kupalov. 1930. Anaerobic and aerobic activity in isolated muscle. Proc. Roy. Soc. B 103:313-322.

Hill, A.V. and P.S. Kupalov. 1930. The vapour pressure of muscle. Proc. Roy. Soc. B 106:445-477.

Hill, A.V., C.N.H. Long and H. Lupton. 1924. Muscular exercise, lactic acid, and the supply and utilization of oxygen. Proc. Roy. Soc. B. 96:438-475.

Hinke, J.A.M. and M.R. Menard. 1976. Intracellular pH of single crustacean muscle fibres by the DMO and electrode methods during acid and alkaline conditions. J. Physiol. 262:533-552.

Hirche, H., H.D. Langohr and U. Wacker. 1970. Lactic acid permeation from skeletal muscle. Pflugers Archiv. 319:R 109.

Hollanders, F.D. 1968. The production of lactic acid by the perfused rat diaphragm. Comp. Biochem. Physiol. 28:907-916.

Huckabee, W.E. 1958. Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion of pyruvate or glucose and of hyperventilation. J. Clin. Invest. 37:244-254.

Huckabee, W.E. 1958. Relationship of pyruvate and lactate during anaerobic metabolism. II. Exercise and formation of O_2-debt. J. Clin. Invest. 37:255-263.

Hutter, O.F. and A.E. Warner. 1967. The pH sensitivity of the chloride conductance of frog skeletal muscle. J. Physiol. 189: 403-425.

Hutter, O.F. and A.E. Warner. 1967. The effect of pH on the ^{36}Cl efflux from frog skeletal muscle. J. Physiol. 189:427-443.

Hutter, O.F. and A.E. Warner. 1967. Action of some foreign cations and anions on the chloride permeability of frog muscle. J. Physiol. 189:445-460.

Irvine, R.O.H., S.J. Saunders, M.D. Milne and M.A. Crawford. 1960. Gradients of potassium and hydrogen ion in potassium-deficient voluntary muscle. Clin. Sci. 20:1-18.

Irving, L., H.C. Foster and J.K.W. Ferguson. 1932. The carbon dioxide dissociation curve of living mammalian muscle. J. Biol. Chem. 95:95-113.

Izutsu, K.T. 1972. Intracellular pH, H ion flux and H ion permeability coefficient in bullfrog toe muscle. J. Physiol. 221:15-27.

Karlsson, J. 1971. Lactate and phosphagen concentrations in working muscle of man. Acta. Physiol. Scand. Suppl. 358:1-72.

Karlsson, J., C.F. Cullie, F. Funderburk, B. Essen and A.R. Lind. 1975. Constituents of human muscle in isometric fatigue. J. Applied Physiol. 38:208-211.

Karlsson, J., S. Rosell, and B. Saltin. 1972. Carbohydrate and fat metabolism in contracting canine skeletal muscle. Pflugers Archiv. 331:57-69.

Karpatkin, S., E. Helmreich and C.F. Cori. 1964. Regulation of glycolysis in muscle. II Effect of stimulation and epinephrine in isolated frog sartorius muscle. J. Biol. Chem. 239:3139-3145.

Kerly, M. and J.H. Ottaway. 1954. The effect of diet on the metabolism of glucose and of acetate by rat diaphragm muscle. J. Physiol. 123:534-552.

Keul, J., E. Doll and D. Deppler. 1972. Energy metabolism of human muscle. Medicine and Sport. 7:134-137.

Khuri, R.N., S.K. Agulian and K.K. Bogharian. 1976. Intracellular bicarbonate of skeletal muscle under different metabolic states. Am. J. Physiol. 230:228-232.

Khuri, R.N., K.K. Bogharian and S.K. Agulian. 1974. Intracellular bicarbonate in single skeletal muscle fibres. Pflugers Arch. 349:285-294.

Kratzing, C.C. 1952. Metabolic effects of electrical stimulation of mammalian tissue in vitro. Biochem. J. 50:253-257.

Lang, E.P. 1934. Observations on lactic acid, total CO_2 and pH of venous blood during recovery from severe exercise. Amer. J. Physiol. 107:687-692.

Lockwood, L.B., D.E. Yoder and M. Zientz. 1965. Lactic acid. Ann. N.Y. Acad. Sci. 119:854-867.

Mainwood, G.W. 1966. Some electrical characteristics of sucrose-washed frog sartorius muscle. Can. J. of Physiol. and Pharmacol. 44:663-674.

Mainwood, G.W. and S.L. Lee. 1968. The hyperpolarizing effect of hydrogen ions on transmembrane potential in frog skeletal muscle. Can. J. Physiol. and Pharmacol. 46:151-157.

Mainwood, G.W. and G.E. Lucier. 1972. Fatigue and recovery in isolated frog sartorius muscles: The effects of bicarbonate concentration and associated potassium loss. Can. J. Physiol. and Pharmacol. 50:132-142.

Mainwood, G.W. and P. Worsley-Brown. 1975. The effects of extracellular pH and buffer concentration on the efflux of lactate from frog sartorius muscle. J. Physiol.

Mainwood, G.W., P. Worsley-Brown and R.A. Paterson. 1972. The metabolic changes in frog sartorius muscles during recovery from fatigue at different extemal bicarbonate concentrations. Can. J. Physiol. and Pharmacol. 50:143-155.

Mansour, T.E. 1963. Studies on heart phosphofructokinase: Purification, inhibition and activation. J. Biol. Chem. 238:2285-2292.

Margaria, R., P. Cerretelli, P.E. Di Prampero, C. Massari and G. Torelli. 1963. Kinetics and mechanism of oxygen debt contraction in man. J. Appl. Physiol. 18:371-377.

Margaria, R., P. Cerretelli and F. Mangil. 1964. Balance and kinetics of anaerobic energy release during strenuous exercise in man. J. Appl. Physiol. 19:623-628.

Margaria, R. and H.T. Edwards. 1934. The removal of lactic acid from the body during recovery from muscular exercise. Amer. J. Physiol. 107:681-686.

Margaria, R., H.T. Edwards and D.B. Dill. 1933. Oxygen debt and lactic acid in muscular contraction. Amer. J. Physiol. 106:689-715.

Miller, R.B., I. Tyson and A.S. Relman. 1963: pH of isolated resting skeletal muscle and its relation to potassium content. Amer. J. Physiol. 204:1048-1054.

Needham, D.M. 1971. Machina Carnis: The biochemistry of muscular contraction in its historical development. Cambridge: Cambridge University Press. pp. 41-42.

Neptune, E.M. Jr., D,R. Foreman and J.J. Reish, Jr. 1960. A requirement for glucose by excised working rat diaphragm. Am. J. Physiol. 199:1048-1050.

Nichols, G., Jr. 1958. Serial changes in tissue carbon dioxide content during acute respiratory acidosis. J. Clin. Invest. 37:1111-1122.

Nissen, P., N. Heisler and J. Piiper. 1972. Buffering properties of red cell suspensions with variations of hematocrit. Pflugers Arch. 338: suppl. R3.

Olson, G.F. 1962. Optimal conditions for the enzymatic determination of L-lactic acid. Clin. Chem. 8:1-10.

Paillard, M. 1972. Direct intracellular pH measurment in rat and crab muscle. J. Physiol. 223:279-319.

Pannier, J.L., J. Weyne and I. Leusen. 1970. Effects of pCO_2, bicarbonate and lactate on the isometric contractions of isolated soleus muscle of the rat. Pflugers Arch. 320: 120-132.

Piiper, J. 1971. Lactic acid formation and buffering in skeletal muscle. Archivio de Fisiologia 68:336-338.

Piiper, J. and P. Spiller. 1970. Repayment of O_2 debt and resynthesis of high-energy phosphates in gastrocnemius muscle of the dog. J. Appl. Physiol. 28:657-662.

Poole-Wilson, P.A. and I.R. Cameron. 1975. ECS, intracellular pH, and electrolytes of cardiac and skeletal muscle. Amer. J. Physiol. 229:1299-1304.

Poole-Wilson, P.A. and I.R. Cameron. 1975. Intracellular pH and K^+ of cardiac and skeletal muscle in acidosis and alkalosis. Amer. J. Physiol. 229:1305-1310.

Prampero, P.E., P. Cerretelli and J. Piiper. 1970. Lactic acid formation in gastrocnemius muscle of the dog and its relation to O_2 debt contraction. Resp. Physiol. 8:347-353.

Roos, A. 1971. Intracellular pH and buffering power of rat muscle. Amer. J. Physiol. 221: 182-188.

Roos, A. 1975. Intracellular pH and distribution of weak acids across cell membranes. A study of D^- and L^-lactate and of DMO in rat diaphragm. J. Physiol. 249:1-25.

Rowlands, S.D. 1969. Oxygen consumption, citrate levels and lactate production of the perfused rat diaphragm. Comp. Biochem. Physiol. 29:1215-1221.

Rowlands, S.D. 1969. The lactic acid production and glycogen content of the perfused rat diaphragm. Comp. Biochem. Physiol. 30:193-208.

Sahlin, K., R.C. Harris, B. Nylind and E. Hultman. 1976. Lactate content and pH in muscle samples obtained after dynamic exercise. Pflugers Arch. 367:143-149.

Schloerb, P.R., G.L. Blackburn and J.J. Grantham. 1967. Carbon dioxide dissociation curve in potassium depletion. Amer. J. Physiol. 212:953-956.

Stainsby, W.N. and H.G. Welch. 1966. Lactate metabolism of contracting dog skeletal muscle *in situ*. Am. J. Physiol. 211:177-183.

Stella, G. 1929. The combination of carbon dioxide with muscle: Its heat of neutralization and its dissociation curve. J. Physiol. 68:49-66.

Strome, D.R. and N.C. Gonzalez. 1975. Bicarbonate transfer as a mechanism of *in vivo* myocardial buffering. Fed. Proc. 34:433.

Thomas, R.C. 1976. Ionic mechanism of the H^+ pump in a snail neurone. Nature 262:54-55.

Tobin, R.B. 1956. Plasma, extracellular and muscle electrolyte responses to acute metabolic acidosis. Am. J. Physiol. 186:131-138.

Trivedi, B. and W.H. Danforth. 1966. Effect of pH on the kinetics of frog muscle phosphofructokinase. J.Biol. Chem. 241:4110-4114.

Turrell, E.S. and S. Robinson. 1942. The acid-base equilibrium of the blood in exercise. Am.J, Physiol. 137:742-745.

Van Slyke, D.D. 1922. On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution. J. Biol. Chem. 52:525-570.

Waddell, W.J. and R.G. Bates. 1969. Intracellular pH. Physiol. Rev. 49:285-329.

Waddell, W.J. and T.C. Butler. 1959. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxaxolidinedione (DMO). Application to skeletal muscle of the dog. J. Clin. Invest. 38:720-729.

Walaas, O. and E. Walaas. 1950. Effect of epinephrine on rat diaphragm. J. Biol. Chem. 187L769-776.

Wallace, W.M. and A.B. Hastings. 1942. The distribution of the bicarbonate ion in mammalian muscle. J. Biol. Chem. 144:637-649.

Wallace, W.M. and O.H. Lowry. 1942. An in vitro study of carbon monoxide equilibria in mammalian muscle. J. Biol. Chem. 144:651-655.

Williams, J.A., C.D. Withrow and D.M. Woodbury. 1971. Effects of CO_2 on transmembrane potentials of rat liver and muscle in vivo. J. Physiol. 215:539-555.

Whaler, B.C. 1960. Some properties of the perfused rat diaphragm. J. Physiol. Lond., 150:15P-16P.

Woodbury, J.W. 1971. As quoted in "Ion Homeostasis of the Brain", B.K. Seisjo and S.C. Sorenson, ed. New York: Academic Press.

Woodbury, J.W. and P.R. Miles. 1973. Anion conductance of frog muscle membranes: one channel, two kinds of pH dependence. J. Gen. Physiol. 62:324-353.