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ADENOSINE AND EXERCISE HYPEREMIA

by

Robert Donald Phair

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Physiology)
in The University of Michigan
1977

Doctoral Committee:

Professor Harvey V. Sparks, Jr., Co-Chairman
Professor John A. Jacquez, Co-Chairman
Professor David F. Bohr
Associate Professor Louis G. D'Alecy
Associate Professor Irving Fox

DEDICATION

"Unless you love someone, nothing else makes any sense."

e e cummings

To Judith, who makes my work make sense.

ACKNOWLEDGMENTS

My dissertation answers several specific questions concerning exercise hyperemia. The experimental work which led to these answers depended to a large extent on the advice and encouragement of my principal thesis advisor, Dr. Harvey Sparks. More important, through his excitement and his willingness to discuss generalizations he has fostered a tremendous maturation of my view of the physical world and my understanding of scientific method.

As co-chairman of my thesis committee, Dr. John Jacquez seemed always to have a valuable suggestion when I faced the innumerable difficulties which attended development of the adenosine assay.

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LIST OF ABBREVIATIONS

$[ado]_{\text{tissue}}$	Adenosine content of tissue sample
$[ado]_{\text{cell}}$	Concentration of adenosine in intracellular water
$[ado]_{\text{ISF}}$	Concentration of adenosine in interstitial fluid
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
free flow	Phrase used to refer to experimental situations in which muscle blood flow is determined by systemic arterial pressure and the conductance of the muscle vasculature
constant flow	Phrase used to refer to experimental situations in which muscle blood flow is determined by a tubing pump on the arterial inflow line

INTRODUCTION

When skeletal muscles contract, their vascular conductance and blood flow increase dramatically. This phenomenon is known as exercise hyperemia; its cause is unknown.

In 1877 Gaskell published observations refuting the hypothesis that extrinsic nerves were required for production of the hyperemia. Since then each new hypothesis has centered on the vasodilator action of some intramuscular factor, either physical or chemical. A great many chemical hypotheses, originating from consideration of the profound metabolic changes in contracting muscle, have been proposed. This is because any molecule is a candidate if it is vasoactive and if its concentration in the environment of the vascular smooth muscle cells is potentially altered during muscle contraction. The large number of metabolites meeting these criteria has prompted the formulation of many specific hypotheses. However, most of the widely circulated hypotheses can now be rejected as explanations of steady state exercise hyperemia (Sparks and Belloni, 1978) although initiation of the hyperemia by one or more of these mechanisms cannot be ruled out (Haddy and Scott, 1968; Sparks and Belloni, 1978).

The present status of many of these hypotheses is described, in capsule form, below.

Release of acetylcholine was one of the earliest explanations to be rejected. Anrep and Saalfeld (1935) showed that exercise hyperemia was unaffected by atropine in doses sufficient to block the response to infused acetylcholine.

Potassium ions (K^+) appearing in the interstitial fluid as a consequence of muscle fiber depolarization have been, on several occasions, proposed as mediators of exercise vasodilation (Dawes, 1941; Kjellmer, 1965; Knochel and Schlein, 1972). Barcroft (1968) has rejected this hypothesis on the grounds that intra-arterial K^+ infusions (Glover et al., 1962) cannot cause blood flow increases comparable to those seen in exercise even when the delivered K^+ concentration is sufficient to cause pain. Furthermore, venous K^+ concentration, although elevated for the first minutes of constant flow exercise, eventually returns to control levels (Morganroth et al., 1975; Stowe et al., 1975; Brace, 1974). This is apparently because the skeletal muscle membrane Na^+-K^+ ATPase is stimulated by high extracellular K^+ , tending to return it to its original level (Hazeyama and Sparks, 1976). Therefore, K^+ must be rejected as the steady state vasodilator; its time course at the site of action is inappropriate.

Changes in tissue osmolality have also been suggested as signals eliciting smooth muscle relaxation during exercise (Stainsby and Fregly, 1968; Mellander et al., 1967; Lundvall, 1972). Although there remains some question about the magnitude of the required osmolal changes compared to those in exercising muscle, osmolality is a stronger candidate than K^+ . This is because a hyperosmolal infusion can maintain dilation for as long as 25 minutes (Stainsby and Fregly, 1968); the effect of infused K^+ wanes within 10 minutes (Kjellmer, 1965). Again, however, the hypothesis must be rejected for the case of steady state vasodilation because elevated venous osmolality,

observed early in constant flow exercise, is not maintained during long-term exercise (Morganroth et al., 1975; Stowe et al., 1975).

Lactic acid production is another metabolic consequence of exercise which prompted formulation of a blood flow regulation hypothesis. The associated decrease in tissue pH is superimposed on the increased acidity secondary to carbon dioxide production. Since infused acid is a vasodilator, Rous and Drury (1929) and Margaria et al. (1933) nominated pH as the mediator. Barcroft (1968) rejected this notion. He contended that

The role of pH, $p\text{CO}_2$ and lactate in exercise hyperemia is probably a minor one. This is indicated by behavior of the circulation in patients with a very rare disease called McArdle's myopathy. In these patients muscular exercise causes hyperemia. But lactic acid is not liberated, nor is there any change in the pH of the venous effluent.

Since CO_2 is itself a dilator (independent of changes in pH) Kontos (1971) has proposed that $p\text{CO}_2$ is an appropriate mediator of exercise vasodilation. He is aware, however, that CO_2 is only weakly vasoactive. Taking this point still further, Stowe et al. (1975) conclude that CO_2 changes alone "...cannot account for most of the vascular response to exercise."

If the end product of oxidative metabolism is not the sought-after signal, perhaps its input is. Guyton and his coworkers (Guyton et al., 1964; Granger and Shepherd, 1973) have championed the hypothesis that oxygen lack is the event which triggers vascular dilation. This claim is made for all situations which result in decreased tissue $p\text{O}_2$. Barcroft (1972) was particularly impressed with their demonstration (Fairchild et al., 1966) that recovery from

reactive hyperemia does not occur in the absence of oxygen. Since he believes active and reactive hyperemias are mediated in the same way, it is not surprising that he is "tempted to think that oxygen lack is the stimulus for vasodilation at the beginning of exercise and is to a large extent responsible for the maintenance of this vasodilation during the steady state."

The strongest argument against the direct action of oxygen is that the pO_2 near the vascular smooth muscle is strongly influenced by diffusion of oxygen from the incoming arterial blood. Using oxygen microelectrodes, Duling and Berne (1970) have shown that periarteriolar pO_2 is only 1.4 mm Hg below arteriolar luminal pO_2 . In addition, he has increased the pO_2 of the solution suffused over the hamster cheek pouch and monitored arteriolar diameter and periarteriolar pO_2 . In the new steady state, vessel diameters were decreased as would be predicted by proponents of oxygen control. However, periarteriolar pO_2 also decreased. From this, Duling (1972) concludes, "...oxygen cannot be directly active in the control of microvessels..."

Still other elements of oxidative metabolism have met the criterion of vasoactivity. Hilton and Chir (1971) have advanced the idea that inorganic phosphate, released during ATP breakdown, escapes from the contracting muscle fibers and causes exercise dilation. Other reports (Dobson et al., 1971; Bockman et al., 1976) have rejected this notion since [phosphate] is not increased in the venous effluent from contracting muscle.

Adenine nucleotides are among the most potent vasodilator

substances known. As such they have been proposed as mediators of exercise dilation even though cell membranes are usually described as being impermeable to them. However, Forrester and Lind (1969) observed an increase in venous ATP after sustained muscular contractions of human forearm. Although they did not identify the source of the nucleotide, their data are consistent with the ATP hypothesis. A further test of this hypothesis by Kjellmer (1965) showed that while infused ATP effectively mimicked the exercise-induced change in capillary filtration coefficient, it also caused a significant relaxation of postcapillary vessels. Since veins are not much relaxed by exercise, ATP was rejected as the vasodilator of exercise.

One major hypothesis, purporting to describe the causal line, even in steady state (> 10 min) exercise, has not been rejected. This one, known as the adenosine hypothesis, has in fact been corroborated in several experimental situations. Its origins can be traced to discovery of the pharmacological actions of adenosine by Drury and Szent-Gyorgyi (1929). They were the first to demonstrate adenosine vasodilation. That it is a potent vasodilator of the skeletal muscle vasculature is demonstrated in several reports, e.g. (Weissel et al., 1973; Dobson et al., 1971).

Within a few years of the Drury-Szent-Gyorgyi paper, Anrep had clearly considered adenosine as an explanation of exercise hyperemia. In his 1935 paper on blood flow and skeletal muscle contraction adenosine is described as a "...recently discovered specific vasodilator substance..." Yet it is inexplicably dismissed:

It is difficult to see how (adenosine) may account for the capillary as well as the arterial vasodilation observed as a result of muscular contraction. At present there is no clear evidence that any of the specific vasodilator substances are formed in physiologically active tissues. Rigler (1932) showed the liberation of some such substance, but his conclusion that this substance was an adenylic compound was made without any direct evidence.

Anrep had also shown that venous blood from a contracting muscle possessed vasodilator activity which was not abolished by partial reoxygenation or by standing (in a "vacuum flask"?!) for 20-30 minutes. This latter observation to yet another premature rejection of the adenosine hypothesis. In 1941 Dawes reasoned that since the vasodilator was stable in blood (Anrep's result) and since enzymes which destroy adenosine had been found in blood, it is "...unlikely that the vasodilator is adenosine..."

Some years after World War II Robert Berne, then at Western Reserve University, became interested in the role played by nucleotides in the regulation of blood flow. His interest may have been stimulated by Rigler's work (1932) (cited twenty years earlier by Anrep). Wolf and Berne (1956) cite Rigler as proposing a "...physiological role (for adenine nucleotides) in the adjustment of blood flow to meet changes in the oxygen requirement of muscle." He does not cite either Dawes (1941) or Anrep (1935). The main results of this 1956 paper are that the adenine nucleotides and their derivatives are potent coronary vasodilators. Adenosine and AMP, however, were only 1/4 as potent as ATP and ADP.

To distinguish among these candidates, Berne pursued the question of whether the highly charged nucleotides can cross cell

membranes. Jacob and Berne (1960) perfused isolated cat hearts with radiolabeled adenosine and concluded, "The myocardial cells appear to be much more permeable to adenosine than to its phosphorylated derivatives, since measurable quantities of nucleotides were not found in the perfusates when the heart was synthesizing ATP from adenosine-8-C¹⁴."

From here it was but a short step to formulation of the adenosine hypothesis. In 1963 Berne reported that when open chest dogs are subjected to severe hypoxia, breakdown products of adenosine (ado) appear in the coronary sinus blood. Since he felt he had shown that ado was a potent vasodilator and that nucleotides were restricted to the intracellular space he was led to postulate that breakdown of adenine nucleotides to ado was not only responsible for hypoxic vasodilation but was "...the physiological agent involved in the intrinsic regulation of coronary blood flow." (Imai et al., 1964). Although Berne and his coworkers were primarily interested in cardiac muscle, they attempted to measure ado in ischemic skeletal muscle as well. While [inosine]_{tissue} increased to ~100 nmol g⁻¹ after 30 minutes of ischemia, and [hypoxanthine]_{tissue} increased to ~20 and ~50 nmol g⁻¹ after these no-flow periods, adenosine could not be detected even after the longest ischemic period. They suggested "...that it is unlikely that adenosine plays a role in the control of skeletal muscle blood flow analogous to that hypothesized for the coronary circulation," but concluded by noting the possibility that "...under physiological conditions quantities of adenosine, undetectable by present methods, may play a role in the regulation of coronary blood flow."

In the absence of sensitive adenosine assay, other means of testing the adenosine hypothesis were used in the mid-1960's. Scott et al. (1965), recognizing that ado is a vasodilator of the canine forelimb vasculature and a constrictor of the renal vasculature, used these beds as a bioassay system for vasoactive molecules in the venous blood from active skeletal muscle. Their data show that contracting skeletal muscle modifies the blood perfusing it (perhaps by addition of a vasoactive substance) in such a way as to cause dilation of the forelimb vessels and constriction of renal vessels. Since AMP and ado were the only known endogenous substances which show this pattern, their observations are consistent with the ado hypothesis.

By 1971 Berne's assay technique was capable of measuring as little as 3 nmol of ado g^{-1} and a reexamination of the role of ado in control of skeletal muscle blood flow was published (Berne et al., 1971). In this study ado was measured by clamp-freezing skeletal muscles of anesthetized rats. $[\text{Ado}]_{\text{tissue}}$ was 4 nmol g^{-1} in control muscles. Twenty minutes of ischemia resulted in an increase to 8 nmol g^{-1} a level similar to that reached (9 nmol g^{-1}) after 20 minutes of 5 Hz contractions with "intact blood supply." Superimposing the 5 Hz contractions and the ischemia for 20 minutes resulted in $[\text{ado}]_{\text{tissue}}$ of 12 nmol g^{-1} . In separate experiments, a fourfold increase in $[\text{ado}]$ was found in the venous blood draining a skinned canine hindlimb subjected to complete ischemia during 5 minutes of tetanic stimulation of the sciatic nerve. All of these results corroborate the adenosine hypothesis for blood flow regulation. While several of these changes are very likely secondary to anoxia, the experiment

involving 20 minutes of free flow 5 Hz exercise must be taken as supporting a role for ado in the mediation of normal exercise hyperemia.

Later in 1971, Berne's group published a full length paper (Dobson et al., 1971), whose purposes included determination of "...the role of adenosine...in the regulation of skeletal muscle blood flow." Using a canine hindlimb, skinned above the ankle and occluded below it, the question was approached by measuring [ado] in arterial and venous blood and in clamp-frozen muscle samples. After taking control samples the hindlimb was stimulated to contract at 20-30 Hz for 5 min with external iliac arterial inflow completely occluded. Experimental muscle samples were obtained from the sartorius muscle during the ischemic contraction and venous plasma samples were collected immediately following its termination. Because the sciatic nerve does not innervate the sartorius muscle the increase (from .7 to 1.5 nmol g⁻¹) in [ado] observed in the muscle samples must be primarily due to the ischemia alone. Furthermore, although the authors say their data "...suggest that adenosine may play a role in the local regulation of skeletal muscle blood flow....," the observed increase (from .3 to 2.3 x 10⁻⁷ M) in common iliac vein [ado] following ischemic contraction must be associated with anoxia in the experimental hindlimb. Consequently these data may be taken as support for adenosine's role in hypoxic or reactive hyperemia but the experiment cannot be taken as an adequate test of the hypothesis that adenosine is the vasodilator of normal exercise hyperemia.

By 1973, Rubio, Berne and Dobson (1973) had carried the comparison of adenosine metabolism in heart and skeletal muscle to the

histochemical level. They found that, in contrast to cardiac muscle, adenine nucleotide degradation in skeletal muscle "...occurs primarily via inosinic acid formation with only a small production of adenosine..." However, their results on skeletal muscle indicated that the adenosine-producing enzyme, 5' nucleotidase, is localized in the vascular endothelium and in muscle fibers near blood vessels. This allowed them to reconcile the differences in AMP degradation with the concept that adenosine regulates blood flow in both heart and skeletal muscle. To supplement this evidence they repeated the experiments on ischemic resting and contracting dog and rat skeletal muscle (Berne et al., 1971; Dobson et al., 1971) and measured tissue levels of adenine nucleotides in addition to adenosine and its breakdown products. All of these results are consistent with adenosine mediation of hypoxic vasodilation; they replicate almost exactly the [ado] measurements reported in 1971 and by repeating the observation of increase tissue [ado] in free or natural flow exercise of rat skeletal muscle corroborate again the hypothesis that ado mediates exercise vasodilation.

In 1972, S. Tominaga became convinced that adenosine was the likely mediator and over the next few years he and his co-workers developed a tracer technique for study of adenosine release. The quantity calculated, which they refer to as "total amount of adenosine/AMP released (TAAR)," is difficult to interpret because it is not explicitly defined. A derivation is presented (Watanabe et al., 1973) but is too imprecise to criticize rigorously. Several interpretations of their results are possible.

Bockman, Berne and Rubio (1975), hoping to avoid degradation of vasodilator metabolites released into blood, measured *ado* in the venous effluent of isolated rat hindlimbs perfused with a buffer solution. Adenosine concentration in the effluent increased with duration of the perfusion, but the increase was greater when 1 Hz contractions for 3 minutes (beginning after 45 minutes of perfusion) were added to the protocol. The hindlimb vascular bed was maximally vasodilated throughout the experiment and was acknowledged to be an unphysiologic preparation. Recognizing that "release of adenosine must be demonstrated under more physiological conditions," these investigators nevertheless conclude that "...adenosine is released when there is a disparity between oxygen supply and oxygen need and that the nucleoside can conceivably be involved in the regulation of skeletal muscle blood flow." This report, which appeared in 1975, provided background for the most direct test to date of the adenosine hypothesis in exercise hyperemia. Published in 1976 by Bockman, Berne, and Rubio, it reports tissue adenosine in clamp-frozen samples of dog skeletal muscle both at rest and during isotonic contraction at rates between 2 and 4 Hz. [Adenosine] in arterial and venous blood was also measured and the hindlimbs were perfused at constant flow to increase the possibility of detecting changes in these variables.

Although the A-V [adenosine] difference did not change in these experiments, [ado] tissue increased from 1.7 nmol g^{-1} at rest to $\sim 8 \text{ nmol g}^{-1}$ during muscle contraction (Bockman, 1976, fig. 2). Their data show a significant increase in [ado] tissue 10 min after onset

of contractions but not after 5 minutes. Since their measurements were made over a period of 25 minutes, they conclude, quite reasonably, that "...adenosine may have a role in the sustained hyperemia associated with skeletal muscle activity."

A pharmacological test of the hypothesis was published by Tabaie, Scott and Haddy (1977). They reasoned that since theophylline can block the vasodilator action of intra-arterial injections of ado, an infusion of theophylline should block that portion of exercise dilation caused by ado. An isolated canine gracilis muscle perfused at constant flow was used for this study. Since theophylline itself has an unexplained vasodilator activity an infused vasoconstrictor (norepinephrine or ADH) was superimposed on the drug infusion to return precontraction perfusion pressure (and vascular conductance) to its pre-drug value. The gracilis nerve was then stimulated at 1 Hz or 6 Hz while perfusion pressure was monitored as an index of vascular resistance.

An infusion of theophylline which blocked the perfusion pressure response to 10 μ g ado also substantially reduced exercise dilation. That this was not due to decreased muscle activity was demonstrated by measuring muscle tension development and venous oxygen tension. Tension development and the exercise-induced fall in venous pO_2 were not changed by theophylline. Absolute venous pO_2 was not reported. These observations led to this conclusion: "...it appears that ado plays an important role in the exercise dilation seen at constant flow in the gracilis muscle of the dog." The paper was concluded by emphasizing "...the need for critical evaluation of the contribution of adenosine to the hyperemia seen under more natural conditions of

exercise." If venous pO_2 had been reported however, we would have some measure of an important potential consequence of constant flow: tissue hypoxia.

To this assessment should be added the reservation that vascular side effects of theophylline (amply demonstrated by its vasodilator action) may account for reduced exercise dilation independent of any anti-adenosine activity.

The gradual approach to assessing the adenosine hypothesis in a physiologically realistic situation has been ordained largely by the slow evolution of assay technology. As the detectable level of adenosine was reduced so could the stress required to achieve that level be reduced. As a consequence of this evolution, however, experimental attention was diverted from the basic physiological question. To date the hypothesis that adenosine, released from muscle fibers, causes an increase in interstitial [ado] followed by arteriolar relaxation remains untested for normal, free flow, exercise hyperemia. Instead the role of adenosine has, for more than 15 years, been evaluated in situations ranging from frank reactive hyperemia to those for which the state of tissue oxygenation can only be guessed.

Historically, the causes of reactive hyperemia and functional hyperemia have often been assumed to be the same. This point of view is well represented by Barcroft's 1972 review lecture. He cites 10 similarities between reactive and active hyperemia, refers to reactive hyperemia as "...a first cousin of exercise," and even characterizes exercise as a "...condition in which the tissues do not get all the oxygen they want." Six years before this lecture,

however, S.M. Hilton (1968) (speaking at a session chaired by Barcroft) noted there was little reason to assume that the same chemical agent was responsible for both hyperemias.

While the mediators of reactive hyperemia and of active hyperemia at constant flow may both be produced in response to transient tissue hypoxia, there is no a priori reason to expect active hyperemia at free flow to be characterized by an equivalent reduction in tissue pO_2 . Hypoxia, through inhibition of oxidative phosphorylation, causes net breakdown of adenine nucleotides and is therefore a potential stimulus to adenosine production which cannot be ruled out in the experiments reviewed above.

Because the most direct test of the hypothesis (Bockman et al., 1976) was carried out with flow restricted to the resting level and without characterization of the state of the sampled muscles ($\dot{V}O_2$, tension development, vascular conductance) and since the animals were not artificially ventilated to maintain blood gases (particularly pO_2) we felt the adenosine hypothesis could be subjected to a more rigorous test.

There are, in the literature, several hypotheses purporting to describe the mechanism by which adenosine production is stimulated during muscle contraction or hypoxia. We will be concerned with these only insofar as they affect delivery of ado to the interstitium. In this sense we will be testing every hypothesis whose result is to increase $[ado]_{ISF}$ during skeletal muscle contraction.

Because our experimental approach relies on tissue biopsy of isolated, in situ skeletal muscle we cannot test hypotheses which

suggest that resistance vessels are controlled by local changes in [ado] secondary to altered metabolism in capillary or arteriolar endothelial cells or in the vascular smooth muscle itself. These concentration changes could occur in such a small fraction of the total muscle mass as to be undetectable in biopsy samples. Furthermore, our test of the hypothesis depends critically on the arguments we will present to establish interstitial adenosine ($[ado]_{ISF}$) as the dominant determinant of our tissue ado ($[ado]_{tissue}$) measurement. (See Discussion.) We also assume that if $[ado]_{ISF}$ increases arteriolar resistance, vessels will relax. This is inferred from the observation that intra-arterially infused adenosine elicits increased blood flow.

The adenosine hypothesis for mediation of exercise vasodilation can, for our purposes, be stated as follows. The release of adenosine from skeletal muscle cells is regulated by cellular mechanisms which cause increased release during muscle contraction. This release transiently exceeds the sum of the rates at which ado is degraded in the interstitial space, is taken up by skeletal muscle and vascular cells and is lost to the vascular compartment by transcapillary diffusion. As a consequence, $[ado]_{ISF}$ increases and arteriolar smooth muscle tone is reduced. This results in increased vascular conductance and allows the experimentally observed increase in blood flow. It is this hypothesis we wish to test.

METHODS

Mongrel dogs of either sex (18-32 kg) were anesthetized with intravenous sodium pentobarbital. The barbiturate was given to effect; the dose was between 25 and 30 mg per kg of dog weight. Maintenance doses were given as required throughout the experimental day.

Each animal was provided with an endotracheal tube so that its respiration could be regulated using a Harvard large animal respirator. Respiratory rate and tidal volume were initially set according to the manufacturer's specifications and were adjusted to regulate arterial $p\text{CO}_2$. Room air at the respirator input was supplemented, as required, with 100% O_2 to adjust arterial $p\text{O}_2$. If necessary, a drip infusion of 1.5% NaHCO_3 was used to correct arterial pH. Blood gases were measured using a Corning Model 165/2 Blood Gas Analyzer. Results of these measurements are shown in Table 1. Heating pads placed under the animal were used to hold esophageal temperature between 38°C and 41°C.

The anterior calf muscles (extensor digitorum longus [EDL] and tibialis cranialis [TC]) of the left hindleg were isolated as described by Mohrman and Sparks (1974); it is outlined here.

Surgery was begun by opening, with electrocautery, the skin of the lateral side of the leg. The peroneus (fibularis longus) muscle was exposed, and its distal tendon was severed. Blood vessels and nerves associated with the muscle were ligated and divided; finally the muscle was removed using the electrocautery gun to separate it at

Table 1: Arterial Blood Gases^a, Hematocrit, Systemic Pressure
and Resting Skeletal Muscle Vascular Conductance

Dog ID	C _O ₂ ^b	pO ₂ ^c	pCO ₂ ^c	pH	Hct	MAP ^c	G _{rest} ^d
<u>2Hz</u>							
12-22-76	18.8	---	31	7.43	53	93	.05
12-30-76	---	---	--	---	--	93	.18
7-26-77	18.0	86	35	7.41	42	132	.16
<u>6Hz</u>							
4-28-77	18.3	85	26	7.44	48	130	.11
5- 6-77	16.7	73	33	7.40	51	130	.15
5-11-77	---	73	33	7.39	47	140	.04
5-17-77	---	104	35	7.44	39	116	.09
<u>Control</u>							
1-12-77	---	---	42	7.37	48	100	.09
1-20-77	15.9	106	35	7.28	48	135	.16
<u>Restricted</u>							
5-25-77	20.4	105	33	7.32	47	145	.11
6- 1-77	20.0	106	39	7.36	47	100	.18
6-15-77	15.6	92	34	7.40	38	132	.30
Mean	18.0	92	34	7.39	46	120	.14
SD	1.8	14	4	.05	5	19	.07

^a mean of samples after adjustments were completed

^b Oxygen content [ml (100 ml blood)⁻¹]

^c mmHg

^d ml min⁻¹ (100 g muscle weight)⁻¹ mmHg⁻¹

its origin. This ends the first stage of the surgery and is done to provide later access to the cranial tibial artery and vein. Next the veins overlaying the distal tendons of EDL and TC were ligated and cut. Thus exposed, the tendons were isolated and freed from both the proximal and distal transverse ligaments. They were then ligated and cut at the level of the distal ligament so that they may later be attached to force transducers. Deep to the EDL tendon the distal portion of the cranial tibial artery was isolated, cannulated for recording perfusion pressure, and separated from the limb below the cannulation point. A bundle containing the two anterior calf muscles as well as their nervous and vascular connections was then discernable when the tendons were pulled away from the limb. This marks the end of stage 2. The next objective is to separate this bundle from the rest of the leg starting at the distal end and working toward its proximal end. Only the proximal tendons and the major vascular connections are preserved. Connective tissue was separated, the fibular nerve innervating TC-EDL was cut, and branches of the cranial tibial artery & vein which perfuse areas other than TC-EDL were ligated and cut as they were exposed. To prevent unmeasured arterial inflow and contaminated venous outflow these branches must be tied almost to the point where the cranial tibial artery emerges from under the tibia and becomes the popliteal artery. Gaining access to these small branches requires removal of the proximal 4 cm of the tibia and excision of the surrounding muscle. This completes stage 3 of the surgery. Stage 4 requires isolation of the popliteal artery and vein near their origins. Access to this point is obtained in the angle

formed by the femur and the caudal border of the gastrocnemius muscle. The popliteal artery arises from the femoral at the same point as the caudal femoral artery and a cannula inserted here can be advanced into the cranial tibial artery as will be done in the last surgical step. Stage 4 was completed by cannulating the contra-lateral femoral artery and vein. The arterial cannula is provided with a T-connector for sampling arterial blood. A flow-through electromagnetic flow probe is also in the line. The outflow end of this cannula is later used to cannulate the popliteal-cranial tibial artery. A venous funnel is attached to the femoral venous cannula. With the exception of polyethylene cannula ends, all extracorporeal tubing is made of Silastic.

At this point heparin (750 U/kg) was administered via the venous funnel and the final stage of surgery was begun. While occluding the popliteal artery, a small polyethylene cannula (PE#280) was introduced into the popliteal vein and advanced into the cranial tibial vein. It was tied in place proximal to the first branch draining the TC-EDL and distal to the last untied branch draining other structures. The arterial occlusion was released and the venous outflow was directed to the previously placed funnel. A portion of the outflow was pumped past a T-connector (for venous sampling) and through an oximeter cuvette before being emptied into the venous funnel. To complete the preparation, the arterial line from the contra-lateral femoral artery was introduced into the popliteal artery, advanced into the cranial tibial artery and tied in place proximal to the first branch perfusing the EDL-TC and distal to the

last untied branch perfusing other tissues. The experimental muscles now have an independent blood supply whose volume delivery rate is easily measured and from which arterial and venous samples are easily obtained.

Arterial perfusion pressure was measured using a Statham Model P23Dc pressure transducer attached to the cannula in the distal cranial tibial artery. This pressure measurement is therefore made downstream from the arterial clamps used in the restricted flow experiments to be described shortly. Calibration was performed as specified by the manufacturer.

The cuvette through which a portion of the cranial tibial venous blood was routed was mounted in a Gilford Model 103-IR Oximeter. The output of this device was calibrated by linear regression of polygraph pen deflection on corresponding values of total venous oxygen content as determined using a Lexington Instruments Corp. oxygen analyzer (Lex-O₂-Con-M).

The muscles were then separated from one another to facilitate the biopsy procedure. A steel rod with a screw tip was driven into the knee joint and clamped to the surgical table. Fixing the proximal ends of the muscles in this way permitted measurement of isometric tension development by tying the distal tendon of each muscle to a special fixture incorporating a Grass FT .03 small displacement force transducer also mounted on the table.

One channel of a Zapeda SWF-3RD electromagnetic flow meter was used with a Zapeda 1/8" flow-through probe (Model #EX 1/8) to monitor blood flow in the arterial line. This device was calibrated by

linear regression of multiple timed collections of venous outflow on the corresponding polygraph pen deflections.

With the exception of esophageal temperature, all continuously monitored variables were recorded on a Grass Model 7D polygraph.

Needle electrodes placed in the proximal and distal ends of the TC-EDL combination were used to elicit muscle contraction. This was accomplished using a Grass Model S48 stimulator to drive a Model SIU5A stimulus isolation unit whose output was applied to the needle electrodes. Constant current pulses of $<.2$ msec duration at frequencies of 2 or 6Hz were applied using a Grass CCU 1A constant current unit. This technique effects twitch contractions by depolarizing the motor nerve endings within the muscle bodies. Autonomic nerve endings are not depolarized using these stimulus parameters.

When desired, two Hoffman screw clamps were placed on the arterial tubing and tightened to restrict arterial inflow to the chosen level. The clamps were placed downstream from the electromagnetic flow probe but upstream from the perfusion pressure cannulation site.

Venous pressure was held at zero (atmospheric) by fixing the outflow orifice of the venous cannula at the same height as the large veins draining the muscles. Vascular conductance of the muscle bed was therefore calculated from blood flow and arterial perfusion pressure. Flow readings were normalized to $\text{ml min}^{-1} (100\text{g muscle})^{-1}$ using the contralateral (control) muscles' weight. They were then divided by perfusion pressure (mmHg). This gives normalized vascular conductance in $\text{ml min}^{-1} (100\text{g})^{-1} \text{mmHg}^{-1}$.

Except during muscle sampling, the preparation was wrapped in Saran wrap to prevent evaporation. Between biopsies the preparation was covered with a gauze sponge soaked in warm saline.

Muscle samples were obtained using a specially designed biopsy punch. The die, similar to a cork borer, but made of 8 gauge (i.d. $\approx$ 3.4 mm) stainless steel tubing, was attached to the drive shaft of a dental drill. This assembly was then mounted on an hydraulic cylinder (Mead) so that when pressure was applied to one side of the cylinder piston the die was driven forward along its axis. The chamber on the other side of the piston was opened to the lumen of the die via copper tubing and a T-connector-bearing combination. When pressure was applied to this side, the hydraulic cylinder was returned to its original position and the contents of the sampling die were rapidly expelled. Pressures of approximately 70 p.s.i. were supplied to each chamber by spring loaded valves each connected to a standard cylinder of 100% N₂ whose output was regulated to $\approx$ 80 p.s.i.

After removing the muscle wrap and positioning the biopsy punch above the isolated muscles, power was applied to the dental drill motor to spin the sampling die. The "forward" valve was then opened and the spinning die driven through the muscle cutting and retaining a cylindrical core of tissue. The tissue sample was immediately blown into liquid nitrogen by directing the die toward the Dewar flask and opening the "reverse" valve. The entire sampling process required between 1 and 2 seconds. Samples were 2-3 mm thick and were stored under liquid nitrogen until completion of the experiment.

Extractions were begun at the end of the experiment. The samples were pulverized under liquid nitrogen and the resulting powder was transferred to tared vials containing 1 ml shell-frozen 6% trichloroacetic acid (TCA). These were stored in a refrigerator overnight. Preliminary experiments showed negligible overnight changes in tare weight using this procedure. Two vials, treated just as the sample vials, but without adding muscle powder, were carried through each experiment as a check.

On the following day reweighing of the vials permitted calculation of the wet weight of muscle tissue in each. Vials were kept refrigerated until weighing and were returned to the refrigerator immediately after the weight was recorded. This procedure was also followed in obtaining tare weights.

The samples were next taken to a cold room (4°C) where the contents of the vials were transferred to cold ground glass homogenizer tubes and pulverized using a motor-driven ground glass pestle. When no particles remained the suspension of denatured protein in acid was poured into small polycarbonate or polypropylene centrifuge tubes. These were spun in a Sorvall (Model #SS-3) centrifuge to sediment the TCA-insoluble material.

After decanting the supernatant into clean glass tubes, its volume was determined by drawing the liquid into a 1 ml blow-out pipet. The muscle extracts were then transferred to numbered centrifuge tubes and extract volumes were recorded. Between .55 and .72 ml was recovered.

Because the enzymes to be used in the purine assay have pH optima which are approximately neutral, 10 M NaOH and other concentrations of both NaOH and HCl were added as required to bring the extract pH into the range between 6 and 8. Determinations of pH were made using a semi-micro electrode and volumes of added base and acid were recorded. These volumes usually totaled less than 0.1 ml.

After overnight refrigeration, the neutralized muscle extracts were assayed for adenosine by spectrophotometric enzyme assay (Kalckar, 1947; Olsson, 1970). This technique measures the uric acid produced as hypoxanthine, inosine and then adenosine are successively degraded by enzyme additions. Xanthine oxidase, nucleoside phosphorylase and adenosine deaminase all have high specificity for their substrates. They were obtained from Boeringher-Mannheim suspended in either glycerol or $(\text{NH}_4)_2 \text{SO}_4$ and were added to the assay cuvet in microliter quantities.

Each cuvet contained .5 ml .2 M potassium phosphate buffer at pH 7. This served not only to hold the pH of the assay mixture near the enzymes' pH optima but also to supply saturating quantities of inorganic phosphate for the nucleoside phosphorylase reaction. In addition, this stabilized two important determinants of molar extinction coefficients: pH and ionic strength.

The volume of the cuvette was then brought to capacity by adding .5 ml of the neutralized muscle extract. The cuvette was covered, then inverted fifteen times for mixing and placed in the sample chamber of an AMINCO-Chance DW-2 dual beam spectrophotometer. The instrument was used in dual beam mode with a deuterium lamp providing the

ultraviolet (UV) radiation source. A high speed "chopper" is employed by the DW-2 to sample the absorbance of two different UV wavelengths. Electrical pulses initiated by this chopper identify the photomultiplier output as either subtrahend or minuend and a virtually continuous record of the difference is plotted versus time by a self-contained X-Y plotter. For this assay the absorbance of the assay mixture at 325 nm was subtracted from its absorbance at 292 nm. Two hundred ninety-two nm is the absorbance peak for uric acid. Using the micromolar extinction coefficient (.0123) for uric acid and the cuvette volume (1 ml), the mass of purine converted to uric acid was obtained. Knowing the volume of acid used to precipitate protein, the mass of muscle collected, the volume of supernatant recovered in extraction, the volume of acid and base used in neutralization and the volume of extract actually assayed permits calculation of the muscle mass represented in the assay cuvette. By forming the ratio of purine mass to muscle mass, the concentration of purine per gram wet weight of muscle was obtained.

Standard adenosine solutions (approximately 100 μ M) were prepared by dissolving 26.7 mg reagent grade adenosine (free base) in 100 ml distilled water. A more accurate measurement of the concentration was then obtained using a Beckman (Model DB) split beam spectrophotometer and a micromolar extinction coefficient (.0154) for adenosine at 260 nm. Each standard was diluted 1:10 before use. Fresh standards were made and assayed in this way for each of several recovery studies undertaken to validate the assay for the conditions in which we used it.

As a preliminary test, known amounts of adenosine (.05 to 1.0 nmol) were added to .5 ml phosphate buffer in an assay cuvette. The balance of the cuvette volume was filled with distilled water and the enzyme assay was carried out as described above. As shown in Figure 1, assay reproducibility was very good and, over the entire assay range, recovered adenosine was very close to the mass of adenosine added.

A more direct test of the assay for the conditions of our experiments was performed as follows. Pieces of skeletal muscle quickly excised from anesthetized dogs were frozen in liquid nitrogen. A relatively homogeneous muscle powder was obtained by pulverizing the pieces under liquid nitrogen and vigorously stirring the resulting powder. Aliquots of the mixed powder (≈ 100 mg) were quickly transferred to tared vials containing 1 ml of shell-frozen 6% TCA, just as described above for experimental samples. The vials were then loosely capped and allowed to thaw in a refrigerator. A volume of diluted standard adenosine solution (usually 20 μ l) was then added to every other vial. This volume was chosen to approximately double the adenosine concentration in the spiked samples. All vials were left in the refrigerator overnight to duplicate the usual experimental procedure. Extractions on the next day and assays on the day after that were performed exactly as described for individual experimental samples.

Background adenosine concentration was assumed equal to the mean concentration in the unspiked samples. Eight such recoveries were attempted. Adenosine recoveries were 69, 104, 112, 108, 93, 137,

Figure 1: Recovery of adenosine standards in water solution. Recovered mass of adenosine versus mass of adenosine added to assay cuvette.

ADENOSINE RECOVERY EXPERIMENTS

ENZYME ASSAY

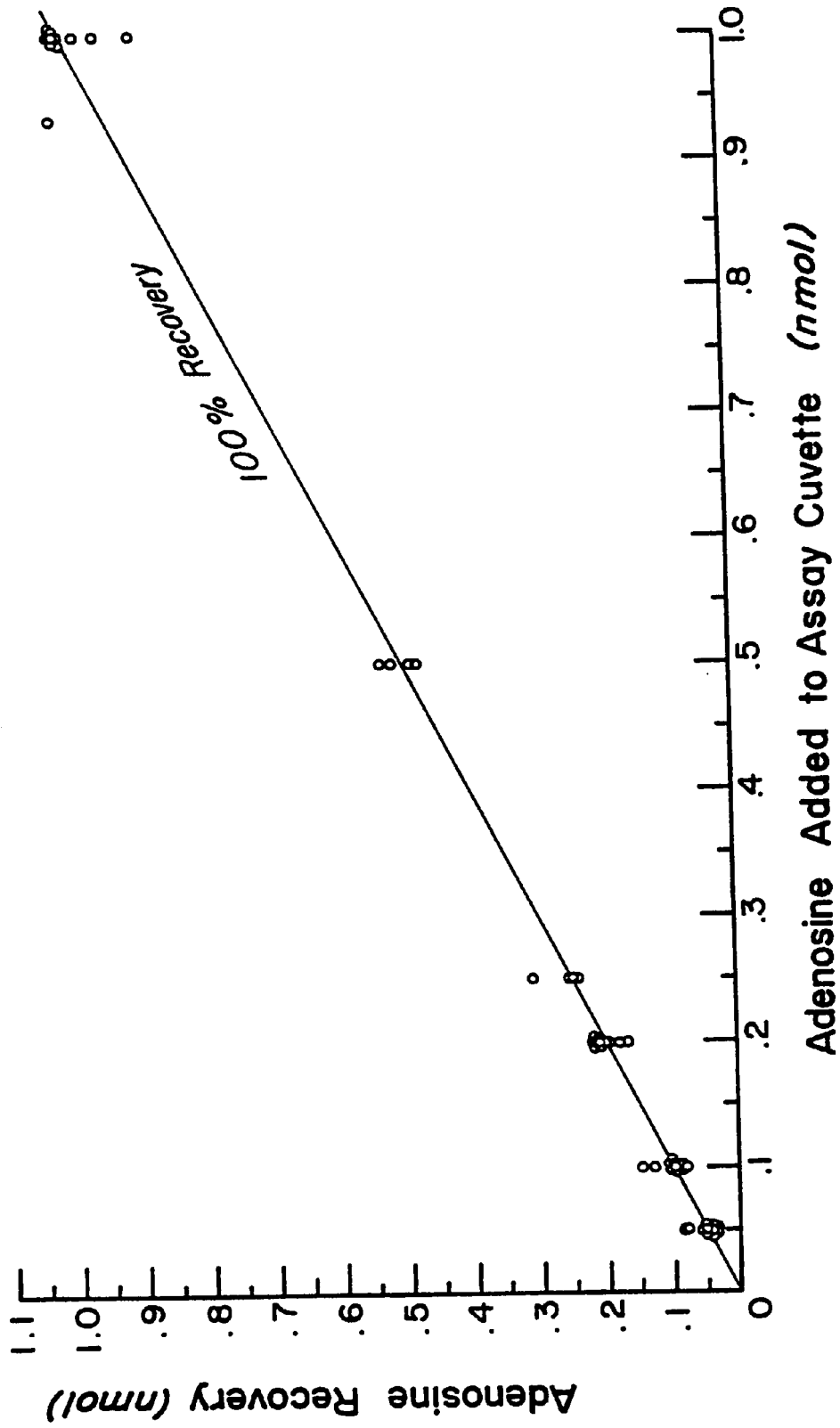


Figure 1

97, and 74%. Thus, in the presence of skeletal muscle extract, we were able to recover $99.2 \pm 2.7\%$ (SE) of added adenosine.

RESULTS

To rule out a possible effect of multiple biopsies on tissue adenosine content we twice ran control experiments in which the isolated muscles were sampled but were not stimulated to contract. Figure 2 demonstrates that no trend toward either increased or decreased $[\text{ado}]_{\text{tissue}}$ was observed. Although two points appeared spuriously high we concluded that the adenosine content of a given sample was not influenced by previous sampling of the same muscle. Since four samples were usually taken from each muscle and since the first sample from the EDL is not different from the last taken from the TC, our assumption of uniform sampling is probably a safe one.

Figure 3 represents our first direct test of the adenosine hypothesis. In three experiments the muscles were stimulated to contract at 2 Hz. Vascular conductance increased from approximately $.1$ to $.4 \text{ ml min}^{-1} 100\text{g}^{-1} \text{ mmHg}^{-1}$. Oxygen consumptions for two of these preparations were 4.5 and $3.2 \text{ ml min}^{-1} 100\text{g}^{-1}$. Although there is considerable scatter in the data, no change was observed in adenosine content of the working muscle even though a steady state hyperemia was established for 30 minutes. Upon cessation of exercise vascular conductance rapidly returned toward its pre-exercise level. Conductance at the end of an experiment usually exceeded the pre-sampling value. This was because sampling opened low-resistance leaks in the vasculature. Post-contraction $[\text{ado}]_{\text{tissue}}$ uniformly fell within the range delimited by the samples taken during

Figure 2: Adenosine content of successive samples of resting skeletal muscle. Tibialis cranialis and extensor digitorum longus muscles prepared just as for hyperemia experiments. Two preparations shown.

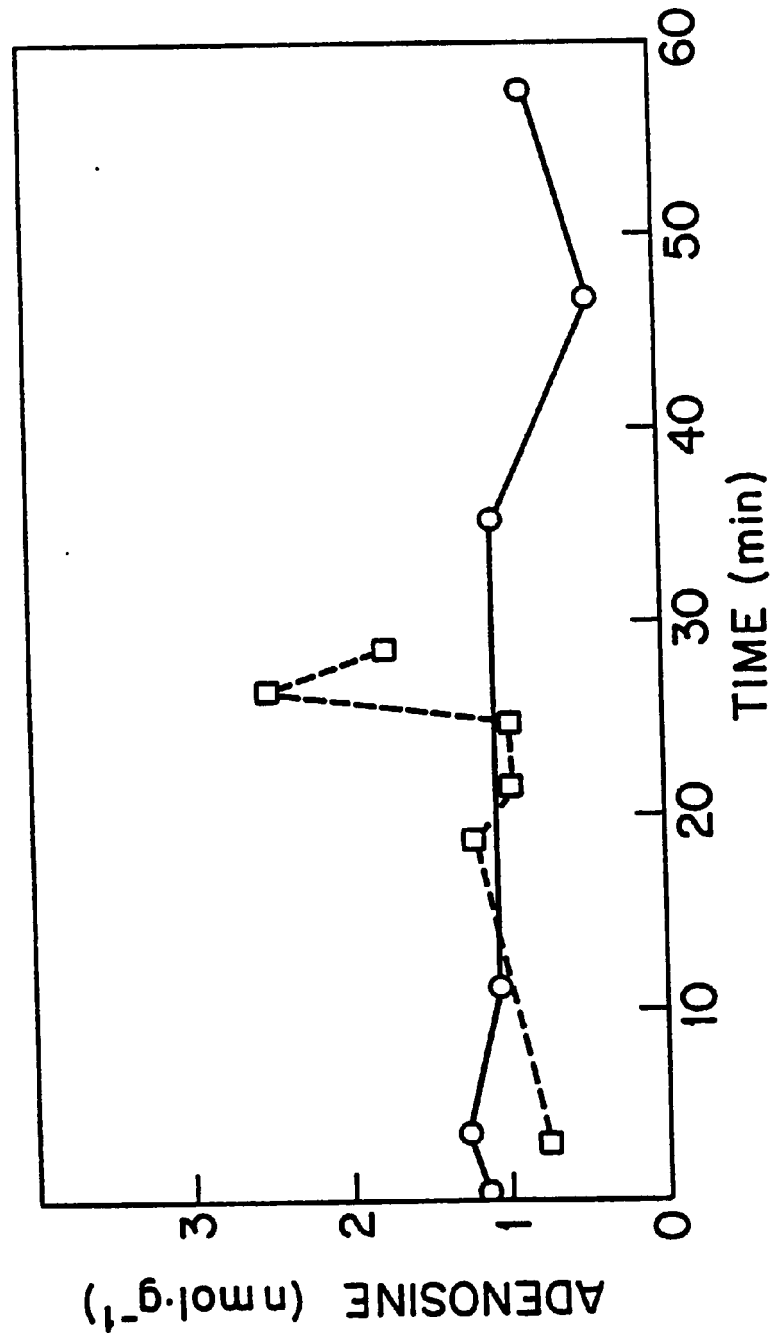


Figure 2

Figure 3: Time courses of vascular conductance and adenosine content of skeletal muscle, before, during and after 2 Hz isometric contractions with free flow. Three preparations.

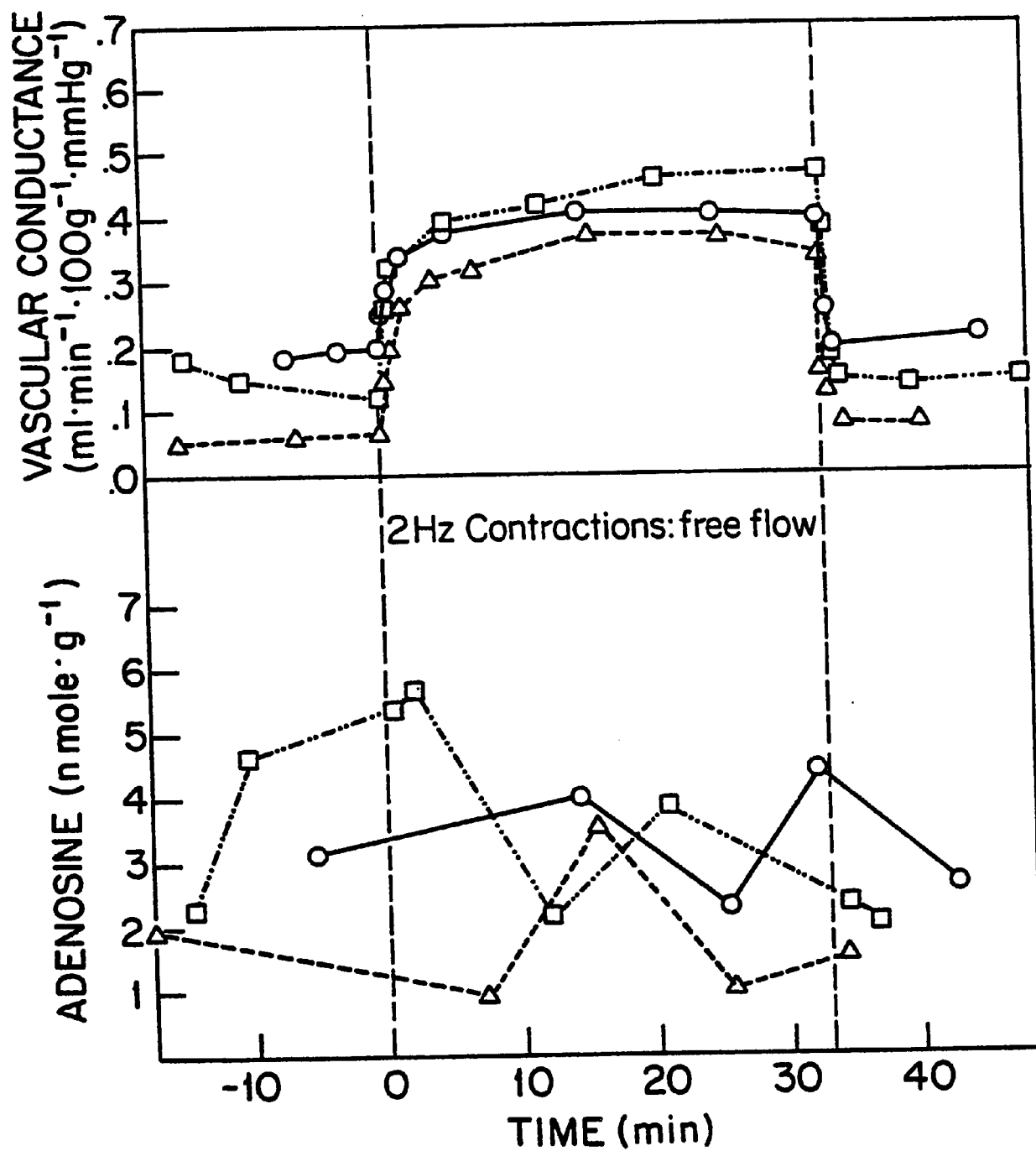


Figure 3

contraction. One experiment was deleted from this series because, after an initial increase, vascular conductance and blood flow decreased substantially during the experimental exercise. This prevented the usual increase in oxygen supply accompanying active hyperemia.

Because tissue pO_2 is such an important determinant of adenine nucleotide metabolism we repeated these experiments using a stimulation frequency of 6 Hz. We reasoned that a higher muscle oxygen consumption might more readily stimulate adenosine production. This was not the case. Figure 4 shows tissue adenosine content and vascular conductance as functions of time during four such experiments. Vascular conductance increased from between .04 and .15 to approximately $.5 \text{ ml min}^{-1} 100\text{g}^{-1} \text{ mmHg}^{-1}$. Again, however, there is no indication of a corresponding increase in tissue adenosine content. Complete information on blood gases was available for two of these preparations. During the steady state of 6 Hz exercise, muscle oxygen consumptions were 7.0 and 7.9 $\text{ml min}^{-1} 100\text{g}^{-1}$. In none of the free flow experiments did tension development decrease by more than 10% as a consequence of the punch biopsy sampling technique.

Together, Figures 3 and 4 demonstrate that tissue adenosine content does not change during experimental exercise at either low or high oxygen consumption. This is reinforced in Table 2. Here, the mean pre-contraction muscle adenosine content for each animal is compared to our best estimate of the corresponding adenosine content during steady state active hyperemia. This estimate was obtained by calculating the average $[\text{ado}]_{\text{tissue}}$ for all samples obtained after

Figure 4: Time courses of vascular conductance and adenosine content of skeletal muscle, before, during and after 6 Hz isometric contractions with free flow. Four preparations.

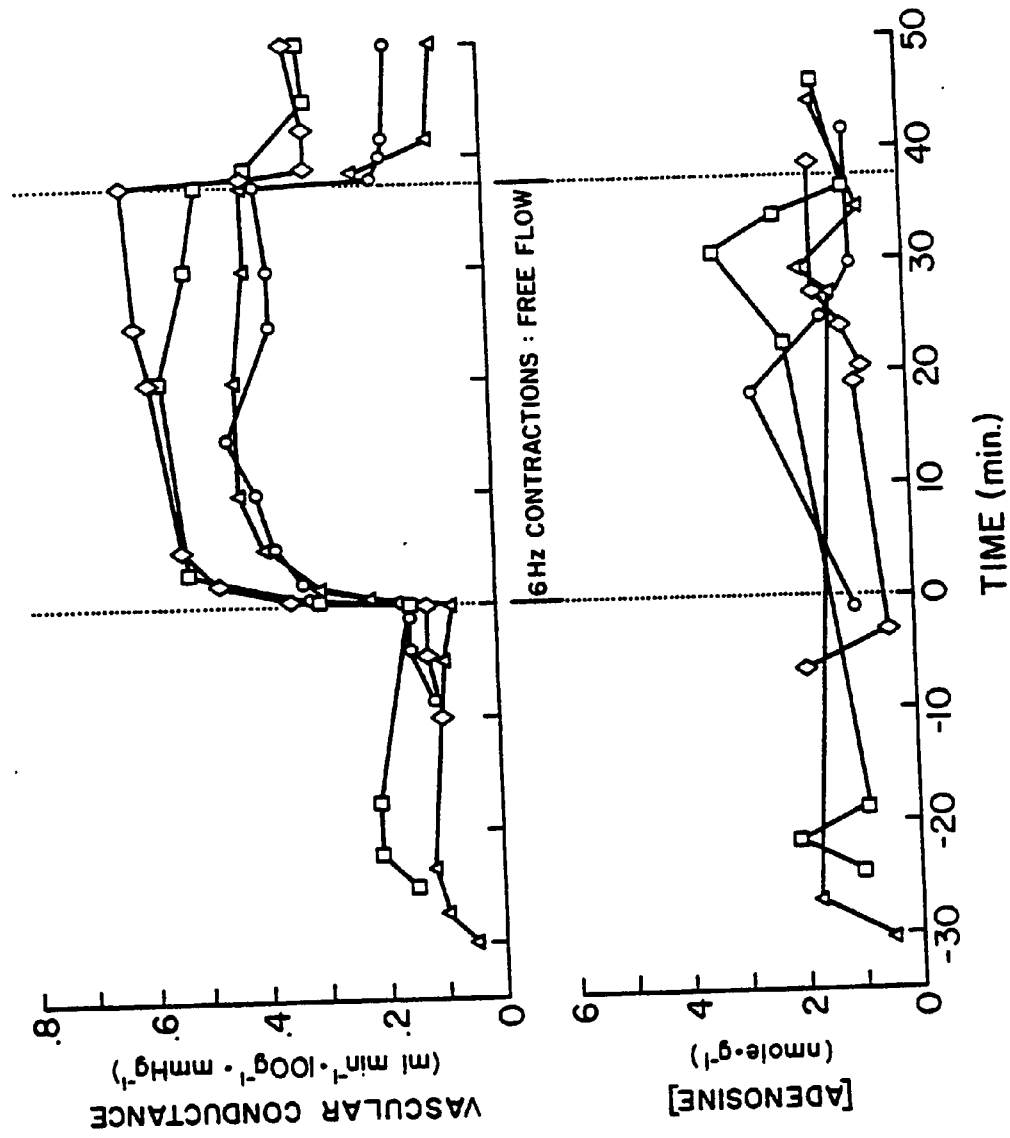


Figure 4

Table 2: Mean adenosine content ([ado]) during precontraction control period and during steady state active hyperemia (between 10 and 30 min. after onset of contraction). Results from three preparations stimulated to contract at 2 Hz and from four preparations at 6 Hz.

<u>2 Hz^a</u>	<u>mean [ado]^b control</u>	<u>mean [ado]^b 10-30 min contraction</u>	<u>Stat^c</u>
12-22	1.93	2.04	
12-30	3.47	3.01	
7-26	3.11	3.58	p>.5
Mean ± SE	2.84 ± .47	2.88 ± .45	
<u>6 Hz^a</u>			
4-28	.92	1.65	
5- 6	1.37	2.17	
5-11	1.11	1.31	.1<p<.2
5-17	1.13	1.07	
Mean ± SE	1.13 ± .09	1.55 ± .24	

^aisometric contraction frequency

^bnmol (g wet weight muscle)⁻¹

^ccompared using 2-tailed paired t statistic

10 minutes of contraction and before contractions were stopped. There is no significant difference between these two measures of adenosine content at either contraction frequency. Therefore, changes in tissue adenosine content, measurable by the technique described here, cannot cause the arteriolar dilation associated with experimental exercise hyperemia.

Furthermore, over the twelvefold range of vascular conductance seen in these experiments $[\text{ado}]_{\text{tissue}}$ changes very little (Figure 5). Indeed, correlation analysis indicates that only 0.4% of the variation in vascular conductance can be explained by a linear dependence on tissue adenosine content.

We postulated that the adenosine production reported by previous investigators was secondary to an insufficient and unphysiological oxygen supply. A final series of experiments was undertaken to test this hypothesis. They were carried out as described for the 6 Hz free flow studies with blood gases adjusted to their normal ranges. After obtaining muscle samples at rest and during free flow exercise, oxygen delivery was experimentally reduced by restricting arterial inflow. As contractions continued muscle samples were obtained at several levels of reduced inflow. The outcome of a typical experiment in this series is shown in Figure 6. In contrast to the free flow experiments (Figure 4), $[\text{ado}]_{\text{tissue}}$ increased (compared to the resting level) as the flow was reduced, reaching 55 nmol g^{-1} during complete ischemia. It should be noted that contractile tension development decreased with each flow reduction but that it recovered if free flow was again allowed. Since a preliminary experiment resulted in

Figure 5: Vascular conductance of skeletal muscle preparations versus the corresponding adenosine content. All points obtained during free flow experiments (Figures 3 and 4) are plotted.

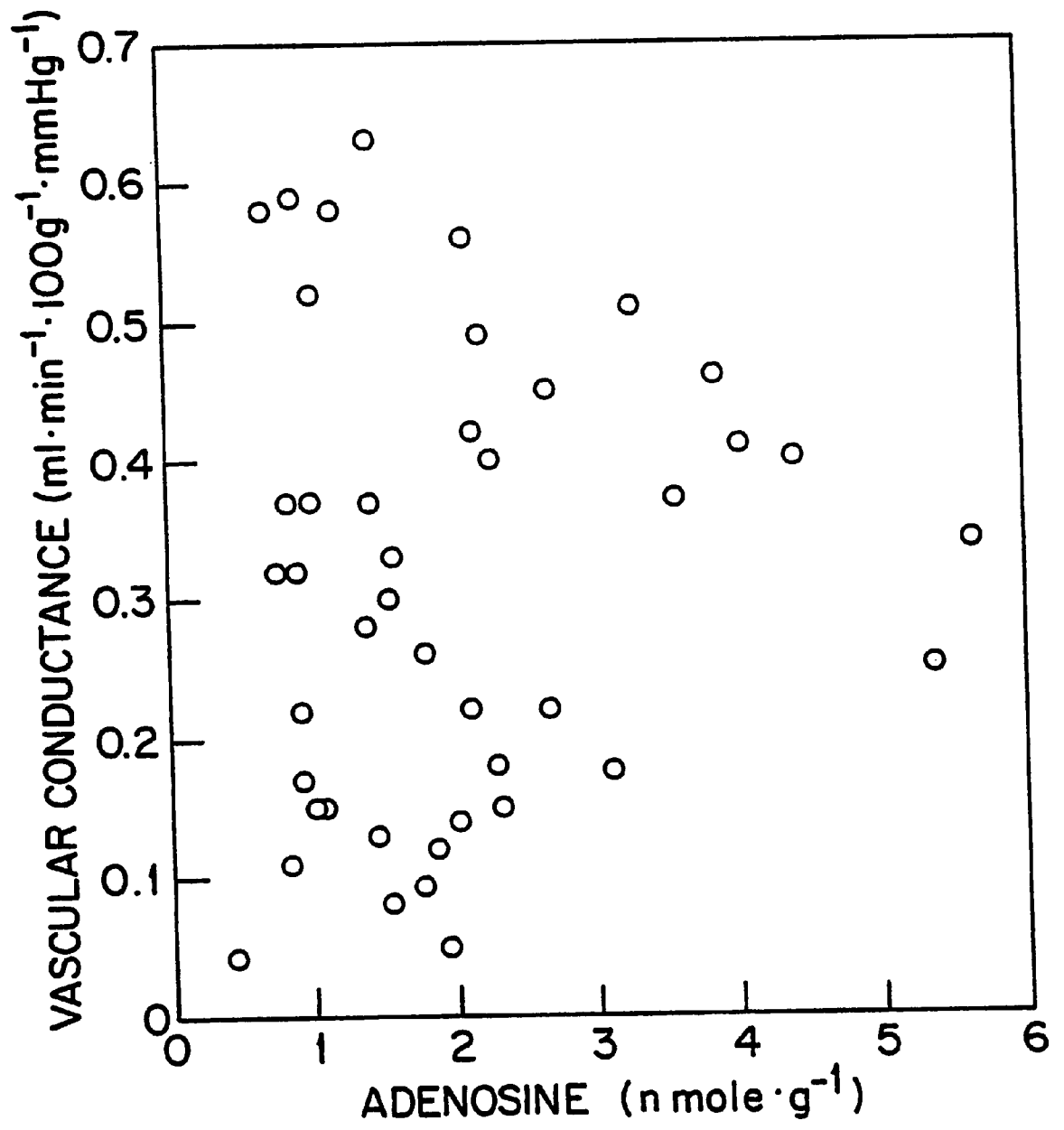


Figure 5

Figure 6: Time courses of arterial blood flow and tissue adenosine content during a typical restricted flow experiment. Step decreases in flow were achieved by tightening adjustable clamps on the arterial cannula. No adenosine measurement during free flow is shown. Duration of 6 Hz contractions indicated by bar.

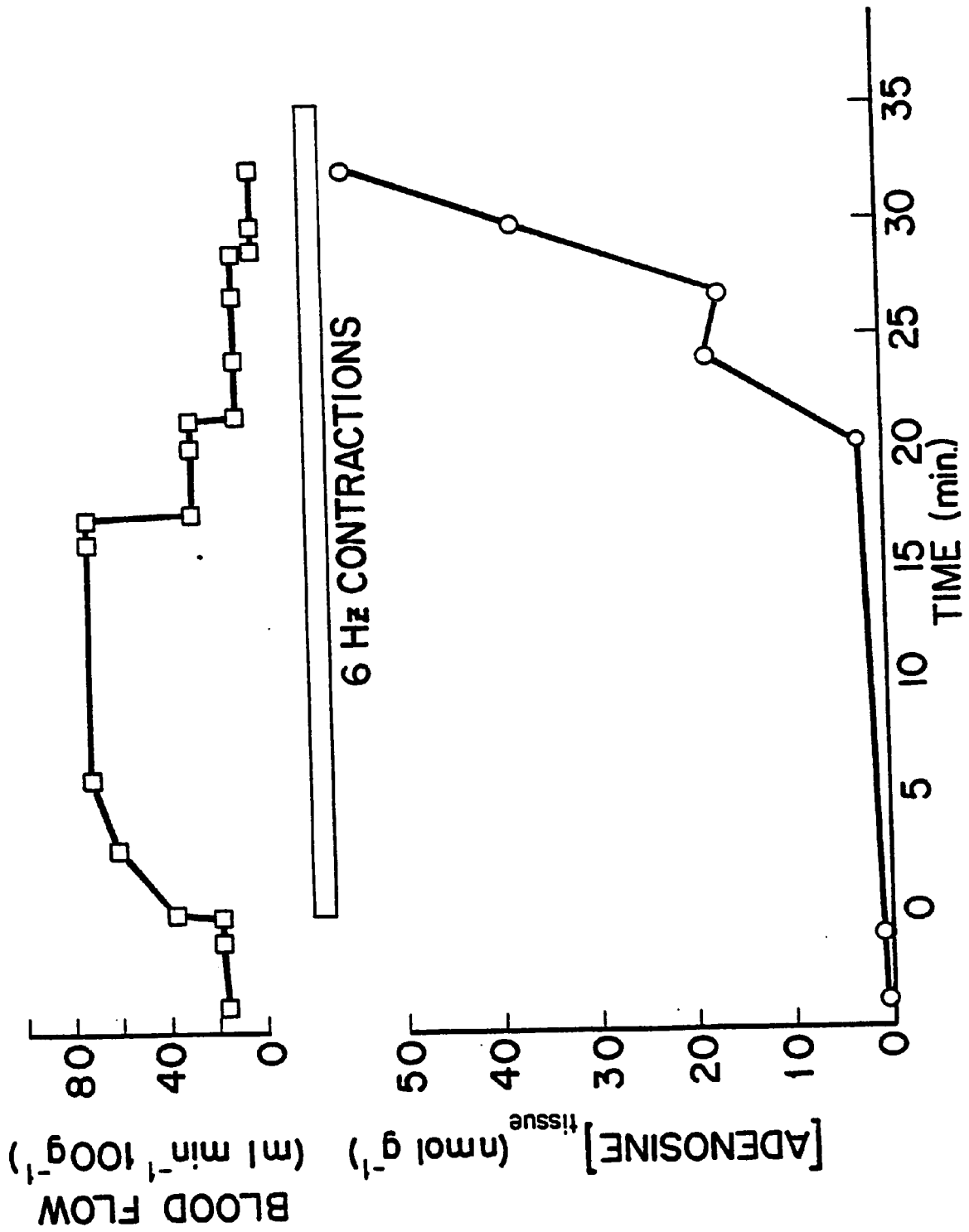


Figure 6

continuously increasing $[\text{ado}]_{\text{tissue}}$ during 2 Hz ischemic contraction, it is unlikely that the restricted-flow concentrations plotted in Figure 6 are steady state measurements. Nevertheless, they demonstrate increases in adenosine content of contracting skeletal muscle caused by arterial blood flow restriction. This result is consistent with our hypothesis and was confirmed in two additional preparations.

By taking oxygen consumption as a measure of "oxygen demand", we can now test the hypothesis, originally proposed by Berne (1963), that an imbalance between the supply of and the demand for oxygen causes (perhaps indirectly) tissue adenosine content to increase. Data from the three experiments like that depicted in Figure 6 are replotted in Figure 7. Tissue adenosine content is on the vertical axis. For each muscle sample the ratio of oxygen consumption to oxygen delivery which obtained at the time of sampling is plotted on the horizontal. This ratio answers the question, "Of the oxygen delivered to the skeletal muscle preparation, what fraction is consumed?"

At rest the answer is less than .15; very little of the oxygen delivered to a resting muscle is consumed. The filled symbols in the lower left of Figure 7 represent resting samples. If adenosine production, stimulated by changes in the oxygen demand/supply ratio, is to play some physiological role one might expect increased $[\text{ado}]_{\text{tissue}}$ as the ratio is increased by metabolic activity. This did not occur. Contractions sufficient to cause consumption of as much as 97% of delivered oxygen did not result in increased $[\text{ado}]_{\text{tissue}}$ when the normal active hyperemia was allowed to occur. During free flow

Figure 7: Adenosine content of skeletal muscle versus the corresponding ratio of muscle oxygen consumption to arterial oxygen delivery. Filled symbols represent points obtained at free flow, open symbols represent samples taken during flow restriction. Symbol shape distinguishes among the three preparations.

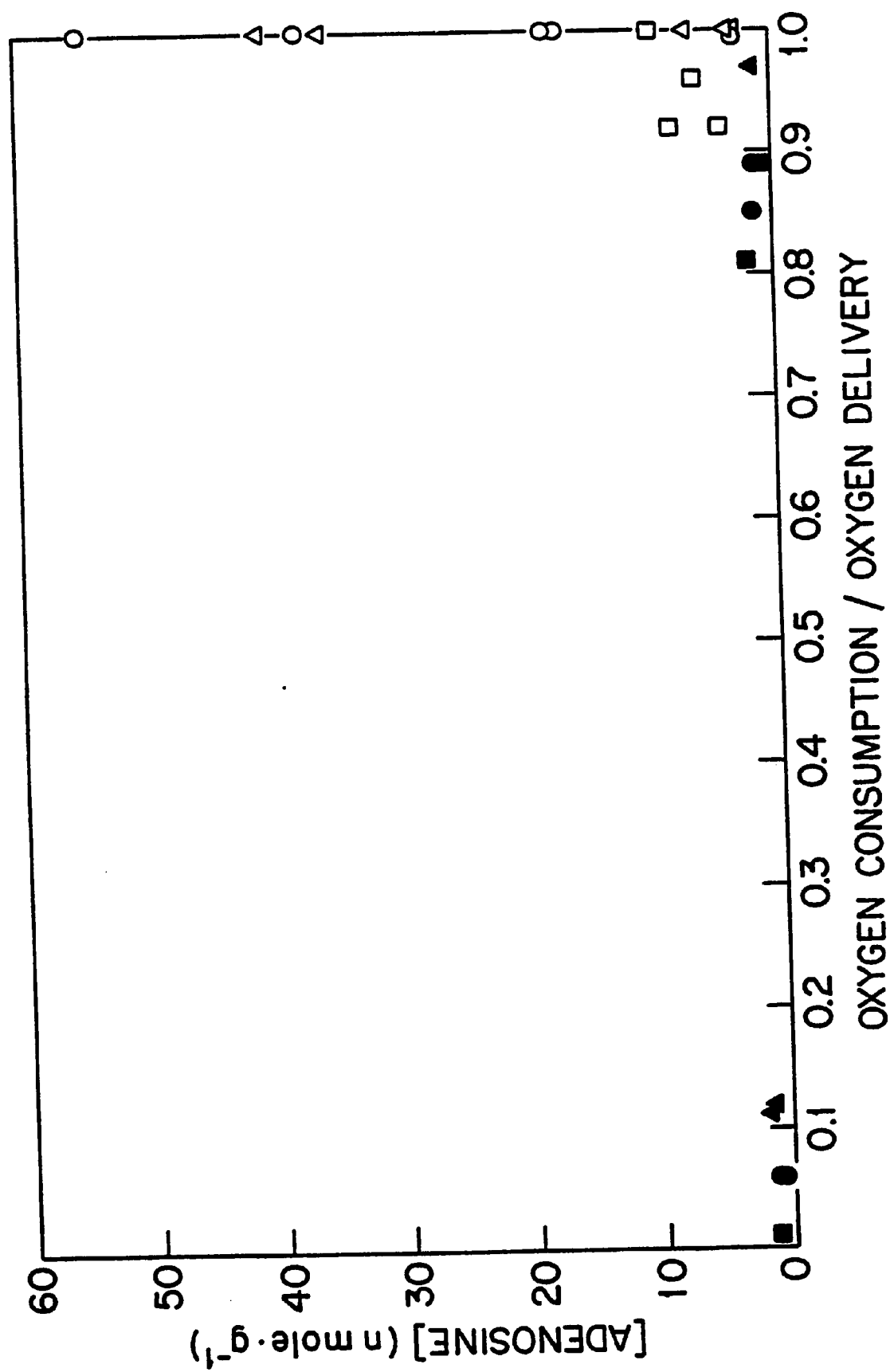


Figure 7

exercise at 6 Hz between 80 and 97% of the oxygen delivered was consumed. The filled symbols in the lower right of Figure 7 represent these samples. Adenosine content under free flow conditions was always less than 2 nmol g^{-1} .

However, when less than the normal hyperemia was allowed, adenosine content increased as the oxygen consumption/supply ratio exceeded 0.9. Very large increases in $[\text{ado}]_{\text{tissue}}$ were seen when oxygen demand was greater than or equal to oxygen supply (ratio = 1.0). The open symbols in Figure 7 represent samples obtained during inflow restriction. Furthermore, as demonstrated in Figure 8, it is only when venous oxygen content drops to 1.4 ml dl^{-1} that tissue adenosine increases. In free flow situations venous oxygen may fall even further without a corresponding increase in $[\text{ado}]_{\text{tissue}}$.

Figure 8: Adenosine content of skeletal muscle versus the corresponding venous oxygen content. Filled symbols represent free flow points, open symbols represent samples taken during restricted flow. Symbol shape distinguishes among the three preparations.

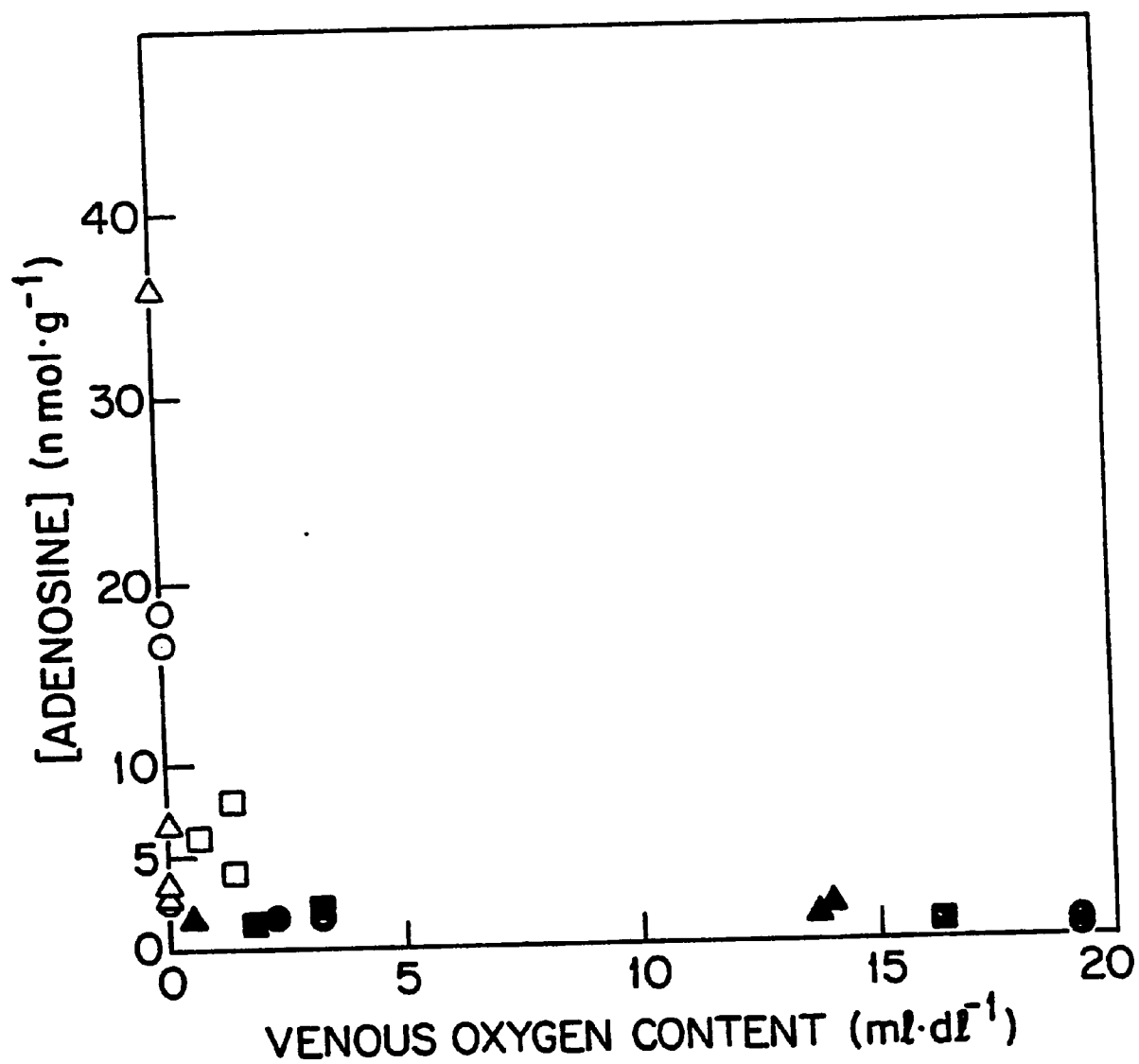


Figure 8

DISCUSSION

The claim of the adenosine hypothesis is that adenosine, formed by the action of 5' nucleotidase, is released from contracting skeletal muscle cells and that once in the interstitium it causes vascular smooth muscle relaxation and increased blood flow. Our test of this hypothesis involved making successive measurements of muscle adenosine content before and during experimental active hyperemia.

We have reported two main results. First, that during sustained 2 Hz and 6 Hz isometric contractions of skeletal muscle, there is no systematic increase in tissue adenosine content. Second, our hypothesis that the adenosine production, seen by previous investigators, was secondary to inadequate tissue oxygenation is supported by our experiments involving progressive restriction of arterial inflow during muscle contraction. Flow restriction limited oxygen delivery to the muscle and allowed us to experimentally increase the ratio of oxygen consumption to oxygen supply. Figure 7 demonstrates that adenosine content does not increase until the ratio attained during free flow exercise is exceeded.

These two results may be interpreted as follows. Under the reasonable assumption that vascular conductance depends only on the present values of its controllers there is an elementary requirement which must be satisfied by any hypothesis purporting to explain exercise hyperemia. Interstitial fluid concentration of the proposed vasoactive substance must be correlated with vascular conductance. As shown in Figure 6, tissue adenosine content is not correlated

with vascular conductance. If our tissue measurement reflects interstitial fluid concentration, the adenosine hypothesis fails this first test.

The second result (Figure 7) suggests a possible relationship between previous reports and ours. The Berne hypothesis is supported, but only outside the range of oxygen consumption/supply ratios attained during changes in muscle activity between the resting state and 6 Hz isometric contraction. In this light, two possibilities might account for the exercise-induced increases in $[\text{ado}]_{\text{tissue}}$ reported by others (Berne et al., 1971; Bockman et al., 1976). First, constant arterial blood flow at the resting level artificially reduces delivery of oxygen to the muscle because the normal functional hyperemia is not permitted. Second, animals anesthetized with pentobarbital are, in our experience, usually hypoxemic unless artificially ventilated. A working hypothesis, consistent with these observations, is that adenosine is produced in response to an imbalance between oxygen supply and demand but that no such imbalance is normally attained in free flow exercise of skeletal muscle.

Taking venous oxygen content as a measure of tissue oxygenation, Figure 8 also demonstrates that restricting flow and holding oxygenation at subnormal levels is an adequate but unphysiological stimulus for increasing adenosine content of contracting muscle. If we assume that free and hemoglobin-bound oxygen are not far from equilibrium at the end of the muscle's exchange vessels, then the plasma pO_2 associated with venous oxygen contents below 2 ml dl^{-1} must be very low. Assuming further that tissue pO_2 is close to equilibrium with

end-capillary pO_2 , tissue pO_2 must be quite low in the restricted flow experiments. It appears likely that oxidative phosphorylation must be compromised before measurable changes occur in tissue adenosine.

Our interpretations of these results depend critically on acceptance of $[ado]_{\text{tissue}}$ as a measure of $[ado]_{\text{ISF}}$. If the reader is convinced that $[ado]_{\text{ISF}}$ is the dominant determinant of $[ado]_{\text{tissue}}$ then the results of this investigation permit rejection of the adenosine hypothesis for mediation of exercise hyperemia at least in our experimental situation. For this reason an examination of the determinants of measured $[ado]_{\text{tissue}}$ forms the next section of this discussion.

Determinants of tissue adenosine content

A discussion of the relationship between measured $[ado]_{\text{tissue}}$ and the $[ado]_{\text{ISF}}$ must answer two major questions: 1) What contribution to $[ado]_{\text{tissue}}$ is made by tissue components other than interstitial fluid? and 2) Can the processes of membrane transport and adenosine metabolism substantially alter $[ado]_{\text{tissue}}$ in the time we required to stop these processes using our biopsy and freezing techniques?

Figure 9 is intended to assist the reader in understanding the calculations which follow. It represents the major physical and chemical pathways of adenosine metabolism in a sample of skeletal muscle.

1) Other Contributions

In addition to interstitial fluid there are two elements of a tissue sample which, potentially, contribute adenosine to the assay

Figure 9: Diagrammatic representation of the major processes and tissue constituents which determine the value of $[\text{ado}]_{\text{tissue}}$ measured in these experiments.

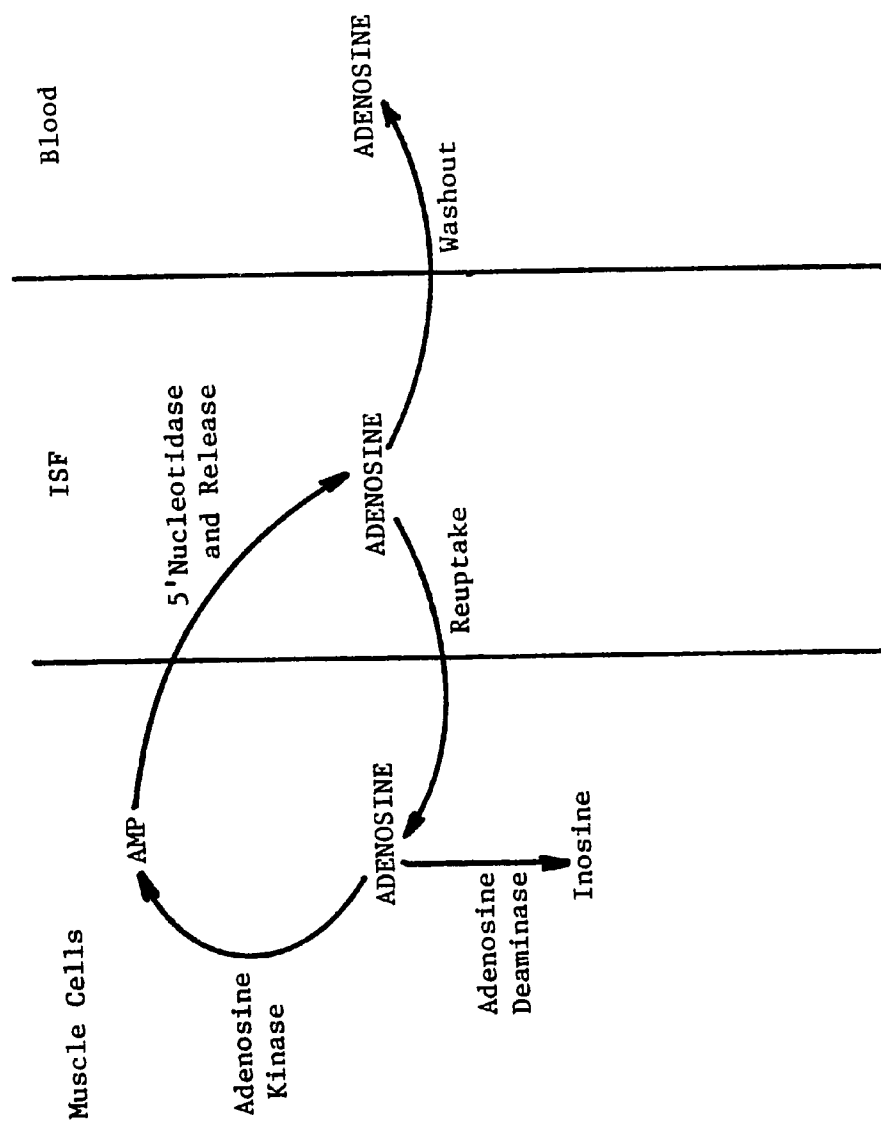


Figure 9.

mixture. These are muscle cells and blood. In the following calculations, the fractions of skeletal muscle mass contributed by muscle cells, interstitial fluid and blood are taken as 0.85, 0.12 and 0.03 respectively. Clearly any free intracellular adenosine will be heavily weighted in a whole-tissue sample and might overwhelm the contributions of interstitial fluid and blood.

a) Contribution of blood and washout of adenosine

An upper limit on the contribution to tissue adenosine made by blood can be obtained by assuming that all of the purines measured in venous plasma after 20 minutes of contraction (Bockman et al., 1976) were present as adenosine before the blood left the tissue. The largest total reported in this paper is $1.2 \times 10^{-9} \text{ mol ml}^{-1}$. In one gram of muscle there are approximately .03 ml of blood. If we assume that the measured plasma concentration also exists in the red blood cells (it is probably much lower because of the action of adenosine deaminase in red cells) we calculate that blood contributes at most $3.6 \times 10^{-11} \text{ moles (mol) of adenosine to each gram of tissue}$. This is two orders of magnitude less than whole tissue adenosine and is therefore negligible even though blood content of muscle increases during exercise.

By taking the reported arterio-venous difference in total plasma purine concentration ($7 \times 10^{-10} \text{ mol ml}^{-1}$) as a measure of adenosine washout and using the muscle plasma flow estimated by Bockman et al. (1976) for their preparation ($.1 \text{ ml min}^{-1} \text{ g}^{-1}$) we can also calculate an upper limit on the magnitude of the washout pathway shown in Figure 9. It is $7 \times 10^{-11} \text{ mol min}^{-1} \text{ g}^{-1}$. This value will be useful

in our consideration of the contribution of intracellular adenosine to $[\text{ado}]_{\text{tissue}}$.

b) Contribution of muscle cells

An upper limit on the value of $[\text{ado}]_{\text{cell}}$ can be calculated by equating the maximum input to the intracellular adenosine compartment with the sum of the adenosine kinase and adenosine deaminase fluxes and solving for the $[\text{ado}]_{\text{cell}}$ which satisfies this steady state requirement. As indicated in Figure 9, it is generally believed that the cellular location of 5' nucleotidase is the plasma membrane and that adenosine, formed by this enzyme, is released directly to the interstitium (arrow labeled "5' nucleotidase and release") (Kohn and Garfinkel, 1977). This limits the input to intracellular adenosine at $4.9 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$, the observed maximal velocity of adenosine uptake (Olsson et al., 1972). A further restriction is imposed by recognizing that the 5' nucleotidase pathway is noncompetitively inhibited by ATP and ADP, making the steady state 5' nucleotidase flux less than the maximal reuptake flux. We will therefore estimate $[\text{ado}]_{\text{cell}}$ by solving the steady state equation for intracellular adenosine using the magnitude of the 5' nucleotidase flux as our estimate of the reuptake flux. The washout flux, calculated in the previous section, is negligible with respect to either the reuptake flux or the 5' nucleotidase flux and is therefore not subtracted from the right hand side. The equation is

$$\frac{V_{\text{max}}^{\text{ADA}} [\text{ado}]_{\text{cell}}}{K_{\text{m}}^{\text{ADA}} + [\text{ado}]_{\text{cell}}} + \frac{V_{\text{max}}^{\text{AK}} [\text{ado}]_{\text{cell}}}{K_{\text{m}}^{\text{AK}} \left[1 + \frac{[\text{ATP}]_{\text{cell}}}{K_{\text{i}}^{\text{AK}} \text{ATP}} \right] + [\text{ado}]_{\text{cell}}} = \frac{V_{\text{max}}^{5\text{N}} ([\text{AMP}]_{\text{cell}} / K_{\text{m}}^{5\text{N}})}{D} \quad (1)$$

where

$$D = \left[\frac{[\text{AMP}]_{\text{cell}}}{K_m^{5N}} + 1 \right] \left[1 + \frac{[\text{ATP}]_{\text{cell}}}{K_i^{5N} \text{ ATP}} + \frac{[\text{ADP}]_{\text{cell}}}{K_i^{5N} \text{ ADP}} \right]$$

Parameter values needed for this calculation are: $V_{\text{max}}^{\text{ADA}} = 1.2 \times 10^{-6} \text{ mol min}^{-1} \text{ g}^{-1}$, $K_m^{\text{ADA}} = 4.3 \times 10^{-5} \text{ M}$, $V_{\text{max}}^{\text{AK}} = 2.3 \times 10^{-8} \text{ mol min}^{-1} \text{ g}^{-1}$, $K_m^{\text{AK}} = 4 \times 10^{-7} \text{ M}$ (Olsson et al., 1972), $K_i^{\text{AK}} \text{ ATP} = 3.3 \times 10^{-4} \text{ M}$ (Kohn and Garfinkel, 1977), $V_{\text{max}}^{5N} = 2.4 \times 10^{-6} \text{ mol min}^{-1} \text{ g}^{-1}$ (Frick and Lowenstein, 1976), $K_m^{5N} = 1.4 \times 10^{-5} \text{ M}$ (Olsson et al., 1973), $K_i^{5N} \text{ ATP} = 1.1 \times 10^{-5} \text{ M}$ (noncompetitive) (Olsson et al., 1973) and $K_i^{5N} \text{ ADP} = 3 \times 10^{-6} \text{ M}$ (noncompetitive) (Kohn and Garfinkel, 1977).

Concentrations of ATP, ADP and AMP in intracellular water were calculated from the data of Rubio et al. (1973); they were $[\text{ATP}]_{\text{cell}} = 7 \times 10^{-3} \text{ M}$, $[\text{ADP}]_{\text{cell}} = 8.1 \times 10^{-4} \text{ M}$ and $[\text{AMP}]_{\text{cell}} = 1.2 \times 10^{-4} \text{ M}$.

Evaluation of the 5' nucleotidase flux gives $2.4 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$ and solution of the equation then gives $[\text{ado}]_{\text{cell}} = 8 \times 10^{-8} \text{ M}$. Assuming there are .6 ml of intracellular water in each gram of muscle, the intracellular contribution to total tissue adenosine is $4.8 \times 10^{-11} \text{ mol g}^{-1}$. This represents only 3.2% of total tissue adenosine. If the remaining tissue adenosine is distributed in the interstitial space $[\text{ado}]_{\text{ISF}}$ must be $1.2 \times 10^{-5} \text{ M}$. This distribution of adenosine is exactly the one required to establish a steady state since adenosine uptake calculated from $[\text{ado}]_{\text{ISF}} = 1.2 \times 10^{-5} \text{ M}$ and the kinetic description of adenosine transport (Olsson et al., 1972) is $2.5 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$ and this is very close to the flux ($2.4 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$) which we have just used to obtain the adenosine distribution. In this steady state

intracellular adenosine makes a negligible contribution to $[\text{ado}]_{\text{tissue}}$.

Notice that this analysis also applies to the situation in which 5' nucleotidase converts intracellular AMP directly to intracellular adenosine rather than releasing the product to the interstitial space. Since washout is negligible with respect to reuptake, we may conclude that the one-way fluxes of adenosine are in balance. This means that the equation (1) is again appropriate and the same negligible contribution to $[\text{ado}]_{\text{tissue}}$ is made by muscle cells in this situation.

If the 5' nucleotidase in skeletal muscle cell membranes is an "ectoenzyme" such as described by Fleit et al. (1975) for lymphocytes, both its substrate pool and its allosteric modulation by ATP and ADP might be different from those assumed above. In this situation, as well as in any experimental situation (e.g. restricted flow) which results in very high $[\text{ado}]_{\text{ISF}}$, input to the intracellular adenosine pool will be limited by the maximal rate of adenosine uptake ($4.9 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$). Equating this to the left hand side of equation (1) does give a larger $[\text{ado}]_{\text{cell}}$ ($1.6 \times 10^{-7} \text{ M}$), but its contribution to $[\text{ado}]_{\text{tissue}}$ ($9.6 \times 10^{-11} \text{ mol g}^{-1}$) is still negligible.

In each case we have established that $[\text{ado}]_{\text{ISF}}$ is the dominant determinant of $[\text{ado}]_{\text{tissue}}$. This provides the required answer to the first major question: What contribution to $[\text{ado}]_{\text{tissue}}$ is made by tissue components other than interstitial fluid? The answer is: negligible contributions are made.

2) Changes with time

Having established, based on enzyme and transport kinetic data, that $[\text{ado}]_{\text{ISF}}$ is the dominant determinant of $[\text{ado}]_{\text{tissue}}$ we are ready to consider the second major question: Can the processes of transport and metabolism substantially alter $[\text{ado}]_{\text{tissue}}$ in the time required to stop these processes?

We required between one and two seconds to perform a biopsy and transfer the muscle sample to liquid nitrogen. The small sample diameter (Lowry, 1953) and the rapid movement of the pressure-propelled sample through the liquid nitrogen (Hoerr, 1936) contribute to stopping the biochemical reactions quickly. The time required is probably short compared to the 1-2 second transfer time.

A worst-case analysis of potential alterations in $[\text{ado}]_{\text{tissue}}$ can be carried out by considering the maximal rates at which adenosine could be removed from or added to the interstitial space. If these maximal rates cannot substantially alter $[\text{ado}]_{\text{ISF}}$ in the 2 sec required to stop reactions, then our $[\text{ado}]_{\text{tissue}}$ measurement is an adequate measure of $[\text{ado}]_{\text{ISF}}$.

Two processes could transfer interstitial adenosine to compartments in which it would be rapidly transformed. These are referred to in Figure 9 as washout and reuptake. Previously noted maximal rates for these processes are $7 \times 10^{-11} \text{ mol min}^{-1} \text{ g}^{-1}$ and $4.9 \times 10^{-9} \text{ mol min}^{-1} \text{ g}^{-1}$ respectively. Together these processes can remove $1.7 \times 10^{-10} \text{ mol g}^{-1}$ in 2 sec if they are unopposed by the process labeled "5' nucleotidase and release" (Figure 9). This is only 11% of total tissue adenosine and can be neglected.

If, on the other hand, the 5' nucleotidase pathway were unopposed by reuptake for the 2 second transfer time, it could add $8 \times 10^{-11} \text{ mol g}^{-1}$ to the sample. This, however, amounts to only 5.3% of tissue adenosine and can also be neglected. We emphasize that these are worst cases; since it is very likely that reuptake will be competing with production by 5' nucleotidase we think large changes in $[\text{ado}]_{\text{tissue}}$ are improbable. The second major question can now be answered. Membrane transport and purine metabolism cannot substantially alter $[\text{ado}]_{\text{tissue}}$ even if allowed to act unopposed for 2 seconds.

In the foregoing analysis we have relied on the accuracy of a model of adenosine metabolism which is based on kinetic data from the experimental literature. We have imposed the requirement for a steady state and have accepted a model only when it was consistent with the experimental data. If we have omitted a significant process in constructing our model, we have also neglected it in evaluating our measurement technique. However, there is excellent agreement between our measured values for resting $[\text{ado}]_{\text{tissue}}$ and those reported by Bockman et al. (1976) who measured adenosine in 500 mg wafers of muscle frozen in situ with aluminum tongs precooled in liquid nitrogen.

Overall, we conclude that our $[\text{ado}]_{\text{tissue}}$ measurement is a good measure of $[\text{ado}]_{\text{ISF}}$. The adenosine hypothesis for mediation of exercise hyperemia must be rejected at least under the conditions of our experiment.

Other Hypotheses

New hypotheses or variants of the original one may be proposed. For example, our experiments could not detect an increase in $[\text{ado}]_{\text{ISF}}$ which falls within the scatter of our data. However such changes are probably not sufficient to cause vasodilation since an infusion which delivers an adenosine concentration (calculated from flow, infusion rate and $[\text{ado}]_{\text{infusate}}$) of 2.8×10^{-7} M causes a 25% flow increase while 3.3×10^{-6} M (calculated in the same way) is required to quadruple resting hind limb blood flow (Dobson et al., 1971). That is, a threefold increase in the blood flow response to infused adenosine required a twelvefold increase in adenosine concentration. In fact, Weissel et al. (1973) required almost an order of magnitude increase in the delivered adenosine concentration (2×10^{-5} M) to elicit the same fourfold increase in hindlimb blood flow.

Another hypothesis which cannot be tested, using our biopsy method, suggests that the vasodilator adenosine pool is formed in the vascular smooth muscle itself. Carotid artery strips in vitro have recently been used (Van Harn et al., 1977) to test this idea. While these investigators did demonstrate adenosine production, it was very small. They concluded, "...it is doubtful that vascular smooth muscle is a significant source of adenosine involved in initiating its own relaxation during anoxia." Even if adenosine were produced by the smooth muscle, this hypothesis would require identification of the physiological signal originating in the contracting muscle fibers and causing the production increase.

A recent paper by Schrader and Gerlach (1976) raises another possible objection to our conclusion. They contend that our calculated $[\text{ado}]_{\text{ISF}}$ of 1.2×10^{-5} M cannot be the concentration to which the vascular smooth muscle is exposed. This argument is based on the adenosine infusion data described in the previous paragraph. It maintains that $[\text{ado}]_{\text{ISF}}$ greater than 10^{-5} M would be associated with blood flows substantially above resting. They conclude that much of the interstitial adenosine is sequestered. Their objection rests on the untested assumption (Dobson et al., 1971) that

...since interstitial fluid and plasma are in communication with the vascular smooth muscle of the resistance vessels, equal concentrations of adenosine in each of these compartments would have comparable effects in attenuating vascular smooth muscle tone.

Several counterarguments can be raised. First, the work of Keatinge has shown that the smooth muscle on the luminal side and on the extravascular side of the vessel wall may be differentially sensitive to vasoactive agents. Second, Dobson's assumption appears untenable given the high adenosine-degradative capacity of vascular endothelium and the non-uniformity of the arteriolar wall. Third, a gradient in $[\text{ado}]_{\text{ISF}}$ (high near the muscle cells and low near the vasculature) is possible. Kohn and Garfinkel (1977) reported that approximately 80% of tissue adenosine deaminase activity must be assigned to vascular tissues in order to account for the large inosine production observed during ischemia. A gradient could be formed if adenosine diffusion toward the vasculature is slow compared to uptake and deamination.

Finally we may evaluate the sequestration hypothesis by considering its consequences. We may begin by assuming that enough of the interstitial adenosine is sequestered to reduce the smooth-muscle-sensed $[\text{ado}]_{\text{ISF}}$ to 1.5×10^{-7} M in a resting muscle. This is a concentration which elicits no vasodilation when delivered to the resistance vessels by infusion. In this situation the contribution of free or unsequestered adenosine to $[\text{ado}]_{\text{tissue}}$ would indeed be negligible. But acceptance of Dobson's hypothesis also requires that $[\text{ado}]_{\text{ISF}}$ increase to approximately 10^{-5} M to account for even a fourfold increase in flow. If this concentration is established by the action of the contracting muscle fibers, it must be established in the entire interstitial fluid compartment. This would add 1.2 nmol g^{-1} to $[\text{ado}]_{\text{tissue}}$, an increase which could be detected using our experimental technique. Furthermore, if the dose response curve to infused adenosine remains linear up to tenfold increases in flow, the required $[\text{ado}]_{\text{ISF}}$ would be approximately 10^{-4} M corresponding to easily detectable increment of 12 nmol g^{-1} . Such large increases in flow are often seen accompanying rapid muscle contraction. Thus we may conclude that if the adenosine hypothesis were correct, even in its "sequestration" form, substantial and detectable increases in $[\text{ado}]_{\text{tissue}}$ would be expected during active hyperemia.

Our overall conclusion is that adenosine, released from contracting skeletal muscle fibers, does not mediate the increases in vascular conductance and blood flow which accompany experimental exercise. This conclusion is of course limited to the conditions of our experiments and therefore applies to denervated red skeletal

muscle in anesthetized and heparinized dogs. On the other hand, our data on the consequences of restricting arterial blood flow to contracting muscle provide support for the hypothesis that adenosine is an important vasodilator substance in hypoxic and reactive hyperemias.

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