EFFECT OF NERVE STIMULATION ON RELEASE OF ADENINE NUCLEOTIDES FROM THE RINGER-PERFUSED CANINE GRACILIS MUSCLE

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
ANN HAVILAND COLLINGSWORTH
1976

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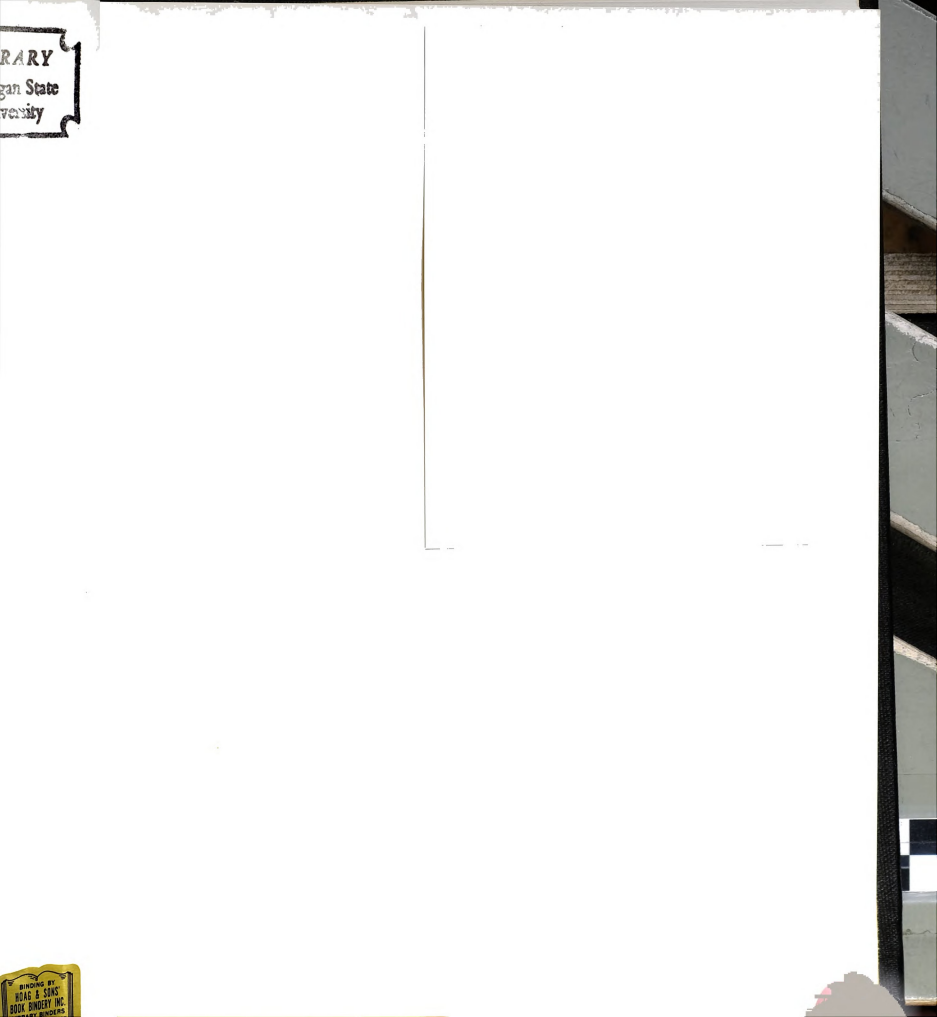

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ABSTRACT

EFFECT OF NERVE STIMULATION ON RELEASE OF ADENINE NUCLEOTIDES FROM THE RINGER-PERFUSED CANINE GRACILIS MUSCLE

By

Ann Haviland Collingsworth

Exercise of skeletal muscle is associated with vascular dilation and an increase in muscle blood flow. The exact mechanism responsible for the vasodilation is uncertain, but it undoubtedly involves a change in the chemical environment of the blood. Since ATP is a potent vasodilator it has been proposed that ATP is the chemical mediator of active hyperemia. The purpose of this study was to investigate the role of adenine nucleotides in exercise hyperemia and in post-occlusion flow changes in the isolated, Ringer-perfused, canine gracilis muscle. ATP was infused intra-arterially into the gracilis muscle, and the effluent was analyzed for ATP content by the firefly-luciferase method. ATP did not survive passage through the muscle vasculature except when infused in large quantities (10 μg/ml or greater). ATP, ADP, and AMP were each infused intraarterially into the muscle, and the effluent was analyzed by gradient elution ion exchange chromatography for breakdown

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products. ATP and ADP were both degraded almost entirely to AMP on a single pass through the muscle. AMP was able to pass through the muscle intact and with little uptake. Therefore, an assay was set up using the myokinase reaction to detect nanogram levels of AMP. Muscular exercise produced by gracilis nerve stimulation at 5V, 2 cps, 1.6 msec caused no changes in venous AMP levels. This was true at the initiation of exercise, during a ten minute period of fatiguing exercise, and during the post-exercise period. A two minute occlusion of muscle perfusion produced no consistent or significant changes in venous AMP. The vasodilator isoproterenol was infused intraarterially to determine if it dilated via AMP release. No significant changes in venous AMP occurred. Vasoconstriction produced by gracilis nerve stimulation at 6V, 6 cps, 1.6 msec or 20V, 10 cps, 1.6 msec was associated with increased outflow of AMP. A curare or gallamine blockade of muscle contraction did not prevent the AMP outflow which occurred with these stimulation parameters. Vasoconstriction produced by intraarterial infusion of epinephrine, norepinephrine, or vasopressin also significantly increased venous AMP levels. The maximal outflow was approximately 150 ng/ml. Phentolamine abolished both the vasoconstriction and the AMP outflow produced by nerve stimulation, epinephrine, and

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norepinephrine. Epinephrine concentrations which did not produce vasoconstriction also did not produce increased AMP outflow. It is concluded that (1) adenine nucleotides probably do not mediate active or reactive hyperemia; (2) isoproterenol does not dilate via release of AMP; and (3) vasoconstriction produced by a variety of agents is associated with increased AMP efflux. The significance of this latter finding is unknown. The source of the venous effluent AMP was not determined.

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EFFECT OF NERVE STIMULATION ON RELEASE OF ADENINE NUCLEOTIDES FROM THE RINGER-PERFUSED CANINE GRACILIS MUSCLE

By

Ann Elizabeth Haviland Collingsworth

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1976

DEDICATION

To my father, Thomas Haviland, and
the loving memory of my mother,
Rosemary.

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I wish to express appreciation to the members of my guidance committee: Drs. C. C. Chou, R. P. Pittman, J. B. Scott, B. H. Selleck, and C. H. Suelter for their time and efforts in my behalf.

The author wishes to thank Mr. George Gamble for his technical assistance. Sincere thanks also go to Mrs. Amylou Davis for her help and advice in many situations where it was much needed.

I wish to especially thank my husband, Carl, for the many hours he spent in preparing all the figures used in this thesis. I also wish to thank my father and brothers for their support and encouragement throughout my education.

Finally, to the many persons in this department who have been helpful in many ways and have added bits of humor to my life during many trying moments I extend my thanks.

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ATP = adenos

ADP = adenos

AMP = adenos

cAMP = cyclic

μCi = microc

c/m = counts

ng = nanogr

kg = kilogr

mg = millig

μg = microg

x G = times

SE = standa

ADH = antidi

P_{O_2} = partia

P_{CO_2} = partia

mm Hg = milli

LIST OF ABBREVIATIONS

ATP = adenosine triphosphate

ADP = adenosine diphosphate

AMP = adenosine monophosphate

cAMP = cyclic adenosine monophosphate

µCi = microcurie

c/m = counts per minuts

ng = nanogram

kg = kilogram

mg = milligram

µg = microgram

x G = times gravity

SE = standard error

ADH = antidiuretic hormone

P_{O_2} = partial pressure of oxygen

P_{CO_2} = partial pressure of carbon dioxide

mm Hg = millimeters of mercury

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INTRODUCTION

Exercise of skeletal muscle is associated with vascular dilation and increased muscle blood flow. The exact mechanism responsible for the vasodilation is uncertain. It has been suggested that a change in the chemical environment of the blood vessels may mediate flow changes. Since adenine nucleotides are present in most cells and are potent vasodilators, the possibility exists that one or more of these compounds may mediate exercise hyperemia. The studies presented in this thesis were designed to investigate this possibility.

The regulation of the peripheral circulation in the intact organism involves adjustments of vascular smooth muscle tone by factors originating from sites both within and outside the tissue the vessels permeate. Synergistic or antagonistic interactions between remote and local control systems combine to determine the level of vascular smooth muscle tone. The remote control system functions in general to maintain systemic pressure and is usually antagonistic to the local control system. The mechanisms of remote control have been fairly well identified and include blood-borne vasoactive hormones, such as epinephrine

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and angiotensin, and sympathetic vasoconstrictor and sympathetic vasodilator fibers. The local control systems attempt to maintain a constant ratio of flow to metabolism in each tissue. The mechanisms of local control have not as yet been clearly identified.

Local control of blood flow includes four phenomena: (1) active hyperemia; (2) reactive hyperemia; (3) autoregulation; and (4) the venous-arteriolar response. All occur in the absence of any external nervous or hormonal influences. Active hyperemia refers to the increased blood flow which accompanies increases in metabolic rate produced, for example, by activation of skeletal muscle. Temporary occlusion of arterial inflow to a vascular bed is usually associated with a transient increase in blood flow above the basal value upon release of the occlusion; this response is reactive hyperemia. Varying the perfusion pressure to most systemic vascular beds over the range 70 to 200 mm Hg results in a less than proportionate change in blood flow. This ability to maintain a relatively constant blood flow despite a varying inflow perfusion pressure is autoregulation. The venous-arteriolar response refers to the arteriolar constriction which results from increases in venous pressure particularly in the intestine and skeletal muscle.

Two major theories have been proposed to explain active and reactive hyperemia. These are the myogenic

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theory and the metabolic theory. The myogenic hypothesis originated with the observation by Bayliss (Bayliss, 1902) that arterial vascular smooth muscle responded to a stretching force by contraction and to a diminution of tension by relaxation. This myogenic activity in response to tension by the smooth muscle cells of small blood vessels has since become an accepted fact. Although its mechanism is unknown (Uchida and Bohr, 1969), distension in some way may increase the instability of smooth muscle cell membranes and thereby lead to depolarization (Wiedeman, 1957). For example, stretch could increase membrane permeability to sodium, thereby producing depolarization. However, considerable evidence exists that the myogenic theory cannot be the sole explanation for either active or reactive hyperemia (Tominaga et al., 1973b; Jones and Berne, 1964). Myogenic mechanisms may be overcome by metabolic demands of the tissue, as suggested by Lundvall et al. (1967).

The metabolic theory of local regulation proposes that blood flow is adjusted to meet metabolic demands of the tissues via alterations in the concentrations of one or more vasodilators in the tissue fluid surrounding the arterioles. Gaskell (1877) was the first to suggest that the blood supply to tissues could be regulated by chemicals affecting their vascular tone. Roy and Brown (1879) found further evidence that blood vessels dilate or constrict in accordance

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with the blood flow requirements of the tissue. They suggested that oxygen or dilator metabolites might play a role in determining vessel diameter. Gaskell (1880) also suggested that intermediary metabolites might be involved in local blood flow regulation, since lactic acid painted on blood vessels produced dilation. Since these early studies, evidence in support of the metabolic theory has accumulated from bioassay studies and chemical analyses of venous effluent. Several excellent reviews of this subject have been published (Ross, 1971; Mellander, 1970; Haddy and Scott, 1968; Haddy and Scott, 1975).

Anrep and von Saalfield (1935) reinfused venous blood from resting and contracting muscles intraarterially into the muscle. Only the venous blood from the actively contracting muscles caused vasodilation. Scott et al. (1965) found that perfusion of the forelimb with venous blood from the vena cava, hindlimb, or heart produced an almost immediate fall in forelimb vascular resistance. The time course and magnitude of the response were similar between assay and donor organ. Vasodilation was produced by venous blood from both resting and exercising tissues, but the dilation was greater when the venous blood was from an exercising muscle. The vasoactive substance or substances appeared to be labile, as the activity of the blood decreased rapidly with time. Selby et al. (1964-65) found

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that venous blood from a previously ischemic bed was vasoactive in the hindlimb. The vasoactivity of venous blood undoubtedly reflects at least an equally great alteration in the vasoactivity of the tissue fluid in the donor organ. Other studies (Rudko et al., 1965) have shown that if the flow-to-metabolism ratio in the canine hindlimb is reduced by either nerve stimulation or obstruction of the femoral artery, vasodilation results. This indicates that the flow-to-metabolism ratio may be an important determinant of vascular resistance, supporting the metabolic theory. Harris and Longnecker (1971) suggested that the metabolic theory is a better explanation than the myogenic theory for pre-capillary sphincter regulation in tissues with a high rate of metabolism, such as the heart. Owen et al. (1975) found the time required for blood flow to return to control levels increased as a function of contraction frequency and exercise duration at both natural and constant flow. Therefore, it would appear that enough evidence exists to seriously consider the metabolic theory in explaining the mechanisms of active and reactive hyperemia.

Much work has been done on the identification of the specific mediator or mediators of the vasoactivity of venous blood. To qualify as a mediator of local regulation, a substance must satisfy several criteria. First, the substance must of course be found normally in the tissue and

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in altered concentration in the tissue fluid or venous effluent during the vascular response. Second, when infused intraarterially, the substance must be vasoactive within the range of plasma effluent or tissue concentrations observed during local regulation. Third, the time course of the concentration changes of the substance must coincide with the local regulatory response.

While no substance has unequivocally met all of these criteria, several substances are still considered to be good candidates for the role of mediator of active and reactive hyperemia. These substances include oxygen, carbon dioxide, hydrogen ions, potassium ions, adenine compounds, inorganic phosphate, and osmolality. Relevant data on each of these candidates regarding active and reactive hyperemia will be discussed in the Literature Review. One further possibility exists, in that more than one of these substances may be altered at the same time. Therefore, it is possible that local regulation is due to the combined effect or interaction of changes in several chemicals.

The purpose of the studies described in this thesis was to determine whether or not adenine nucleotides are chemical mediators participating in active and reactive hyperemia in canine skeletal muscle. Forrester and Lind (1969) and Chen et al. (1972b) found ATP released into the venous effluent from exercising skeletal muscle. However,

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Kontos et al. (1968a) and Dobson et al. (1971) reported no change in ATP in the venous blood draining exercising muscle. Therefore, further studies in this area appeared necessary. The experiments to be described were all performed in the artificially perfused gracilis muscle to eliminate the rapid breakdown of adenine compounds which normally occurs in whole blood and plasma (Collingsworth et al., 1974).

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LITERATURE REVIEW

1. Active Hyperemia of Skeletal Muscle

This section reviews the evidence for the roles of potassium, oxygen, carbon dioxide, hydrogen ions, osmolality, adenine compounds, and several other factors in active hyperemia in skeletal muscle. Studies are included which show whether or not vasodilation occurs when the concentrations of these chemicals are altered in the arterial inflow of resting muscle. In addition, the evidence for the participation and interaction of these factors in exercise hyperemia is reviewed.

Potassium. Many studies have been done to determine the effect of potassium on vascular resistance. Dawes (1941) was the first to study potassium in detail; he suggested that it might mediate exercise hyperemia. In adrenalectomized whole cats or in the perfused hindlimbs of dogs and cats, small doses (5 mg) of potassium chloride infused intraarterially produced vasodilation whereas large doses (20 mg) produced vasoconstriction. Glover et al. (1962) confirmed the vasodilator action of potassium in humans. Emanuel et al. (1959) noted vasodilation in the arterioles of the dog forelimb with infusions of hyperosmotic potassium

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solutions up to a concentration of eight meq per liter. Above this concentration vasoconstriction occurred in both arteries and arterioles. The vasodilation still occurred after denervation and adrenergic blockade with phentolamine, suggesting that potassium dilates via a direct action on vascular smooth muscle. During the potassium-induced vasodilation, responses to norepinephrine were diminished. This effect was also noted by Frohlich et al. (1962) with potassium infusion at concentrations too low to produce vasodilation. Konold et al. (1968a) also confirmed this sympatholytic effect of potassium; venous resistance was not affected by any of the potassium concentrations. Haddy et al. (1963) and Roth et al. (1969) found vasoconstriction when the normal potassium concentration in plasma was reduced toward zero.

Overbeck et al. (1961) confirmed that the vasodilator action was not due to hypertonicity of the infusion solutions. Infusion of isotonic solutions of potassium salts into the dog forelimb produced vasodilation, indicating that previous vasodilation seen with hypertonic potassium salt infusions was indeed due at least in part to potassium and not solely to hypertonicity. Lowe and Thompson (1962) also found that the vasodilator action of potassium was probably a direct one. The dilation produced by infusion of isotonic potassium chloride solutions into the human

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forearm was not blocked by atropine or a β-receptor blocking agent. Baetjer (1934) found that potassium-induced dilation was not affected by curare. With isolated bovine facial arteries Konold et al. (1968b) found that tetrodotoxin did not block potassium-induced vasodilation, suggesting that local nerves are not involved. In further studies (Konold et al., 1968c) these workers found that rinsing the external surface of isolated bovine facial arteries with potassium chloride solutions produced the same vasodilator effect as infusions had. This further supported a role for potassium in exercise hyperemia, as the released potassium presumably would enter the vessels from the tissue fluid.

The mechanism of potassium-induced resistance changes has been studied by several investigators. Chen et al. (1972a) found that the normal response of the isolated canine gracilis muscle to both hyperkalemia and hypokalemia was blocked or reversed by pre-treatment with the sodium pump inhibitor ouabain. This suggested that the vasodilator action of potassium was related to stimulation of membrane Na^+-K^+-ATPase, resulting in hyperpolarization and relaxation of the vascular smooth muscle cell. In further studies (Brace et al., 1973) the time course of the resistance changes produced by potassium was studied. Hyperkalemia produced an initial decline in resistance, followed by a gradual increase in resistance to values above control

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levels in five minutes. The time course closely paralleled changes in resting membrane potential predicted by a computer model of a vascular smooth muscle cell. It was suggested that the initial dilation was due to stimulation of the electrogenic sodium-potassium pump, whereas the secondary constriction was due to the effect of changes in intracellular sodium on the pump. Anderson (1976) presents an excellent review of this subject.

Gebert et al. (1969) investigated the mechanism of the response of isolated arteries to potassium. Depleting muscle cells of potassium by cooling did not prevent the vasodilator response to increased extracellular potassium. The effect of potassium on the membrane potential was measured by means of the sucrose gap technique. Dilating concentrations of potassium sulfate and potassium chloride produced very small or no depolarization. ^{42}K-labelled arterial strips did not show increased potassium efflux in the presence of dilating concentrations of potassium chloride in the external medium. Due to this evidence an alternate hypothesis for the mechanism of potassium vasodilation was offered. It was suggested that potassium might stimulate cyclic AMP for the following reasons. Cyclic AMP produced dilation, and even after cold storage the ATP content of the muscle cells was high enough for cyclic AMP formation. The phosphodiesterase inhibitor theophylline

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also produced vasodilation. Thus, it was considered possible that cyclic AMP mediates potassium vasodilation.

Baetjer (1934) was one of the first to report increased plasma potassium following muscular exercise in cats produced by somatic nerve stimulation. Sympathetic nerve stimulation did not consistently produce increased plasma potassium. Fenn (1937) confirmed these results in the rat and frog. Kjellmer (1961), in cat calf muscles, noted increased potassium levels in venous plasma during exercise and for some time afterwards. The increase could be imitated by intraarterial infusions of isotonic potassium salts. However, the infusions only produced one-third of the vasodilation seen during exercise. Assuming that interstitial fluid potassium concentration during exercise would be higher than plasma potassium concentration, approximately two-thirds of exercise vasodilation could be accounted for by locally released potassium. In addition, it was demonstrated that both exercise and potassium infusion probably affect the same vascular sections, as rate of flow, increase in regional blood volume, and opening up of additional capillaries were well correlated between the two cases. Kjellmer (1965) further confirmed these results, finding up to a 100% increase in venous plasma potassium during calf muscle contractions. Potassium infusions mimicked the vascular response to exercise: decreased flow

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Laurell and Pernow (1966) found that the elevated plasma potassium levels following exercise in humans fell rapidly after the exercise, reaching control levels within 30 seconds. Other parameters, such as pH and lactate, did not return to normal levels as quickly. The arterio-deep venous concentration difference in potassium was much greater than the arterio-superficial venous difference. Scott et al. (1970) found that in the dog hindlimb and gracilis muscle the venous potassium concentration rose at once with exercise and remained elevated during five minutes of exercise. In the cat gastrocnemius 20% of maximal potassium release was associated with 20 to 70% of maximal vasodilation (Hilton and Hudlicka, 1971). Further increases in potassium produced only small increases in blood flow. In the cat soleus there was no relationship between potassium release and exercise hyperemia. Kjellmer (1960) reported that in cat calf muscles no exercise hyperemia occurred during a potassium-induced vasoconstriction, although the response to injected vasodilators was not blocked. Norepinephrine-induced vasoconstriction did not prevent exercise hyperemia. Knochel and Schlein (1971) found that in canine gracilis muscles which had been potassium-depleted

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by 25% the vasodilator response to exercise was much diminished. A 10% depletion had no effect on exercise dilation (Anderson et al., 1972).

Chen et al. (1972a) found that vasodilation still accompanied exercise after pre-treatment of the gracilis muscle with ouabain. However, two changes were apparent in the vasodilator response. After ouabain the onset of the dilation was delayed, and the time required for perfusion pressure to reach a minimum stable level was prolonged. Since ouabain blocked the dilator response to hyperkalemia, it was suggested that potassium probably does have an important role in the initiation of exercise hyperemia. Further evidence that potassium may cause the initial dilation of exercise was provided by Mohrman and Abbrecht (1973). Following a very brief tetanus in dog muscle, there was a transient increase in venous plasma potassium. The increase occurred rapidly enough to account for the vasodilation which immediately occurred. Tominaga et al. (1973a) noted elevated venous plasma potassium at the start of muscular contractions in the canine hindlimb. The potassium remained elevated during an eight minute period of exercise. However, potassium levels rapidly returned to control following cessation of stimulation and showed no correlation with vascular resistance. These results were confirmed by Morganroth et al. (1974).

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Several workers have published data showing that potassium cannot be the important mediator of the maintained response to exercise. Anderson et al. (1972) changed the level of potassium in perfusing blood from normal to hypokalemic during the dilation of simulated exercise in canine skeletal muscle. No change in vascular resistance occurred. Also, the magnitude of exercise resistance changes were much greater than could be induced by local changes in plasma potassium alone. Only a 10% change in resistance could be accounted for by potassium, whereas a 32% resistance decrease occurred. Radawski et al. (1972b) found potassium levels in the venous plasma from the exercising gracilis muscle only slightly above arterial levels after two hours of stimulation.

The source of the potassium efflux during exercise appears to be skeletal muscle. Sreter (1963) found progressive depletion of intramuscular potassium with prolonged exercise. Scott et al. (1970) found a rise in venous effluent plasma potassium concentration during exercise while perfusing with red cell-free fluids. Also, potassium increases were exaggerated with constant flow. In addition, dog red cells have a potassium concentration similar to plasma. Kilburn (1966) observed no change in erythrocyte potassium levels during exercise. Since both potassium and hydrogen ion levels were elevated in the venous plasma from

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exercising human muscles, it was suggested that the mechanism of the potassium increase was an exchange of hydrogen ions for muscle cell potassium ions. Hypoxia and acidosis, both of which are believed to occur during prolonged exercise, would increase potassium leakage from muscle fibers. In addition, a very small decrease (0.5%) in muscle fiber potassium would produce a large increase in extracellular potassium. The evidence therefore indicates that muscle fibers are the source of the potassium efflux observed during exercise.

Oxygen. Several early studies suggested a connection between blood flow and tissue oxygen uptake. Verzar (1912) reported that decreasing the flow to resting cat skeletal muscle decreased oxygen uptake, although venous blood from that muscle still contained substantial amounts of oxygen. He suggested that oxygen consumption is limited by the blood flow. Pappenheimer (1941a) noted that muscle blood flow was directly proportional to its rate of oxygen consumption over a considerable range. Hudlicka and Vrbova (1958) showed that oxygen consumption increased to five times control during muscle stimulation.

Several workers have reported the effects of oxygen on skeletal muscle vasculature. Blood flow in the canine hindlimb increased to three times the normal level as arterial oxygen saturation declined from 100% to zero (Ross et al.,

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1962; Attinger et al., 1967). Daugherty et al. (1967) reported that only P_{O_2} values below 40 mm Hg caused vasodilation in the canine hindlimb. Alterations of oxygen content over the range of normal arteriovenous differences had little effect on dog forelimb and hindlimb vascular beds (Molnar et al., 1962). Guyton et al. (1964) demonstrated that carbon monoxide or cyanide poisoning produced total-body vasodilation. Perfusing isolated small arteries from muscle with bloods in which P_{O_2} was decreased from 100 to 30 mm Hg caused progressive vasodilation. The arteries showed extreme conductance changes even in the normal tissue P_{O_2} range. Carrier et al. (1964) confirmed these latter results and found that smaller vessels were much more sensitive to decreases in P_{O_2} than larger ones. Results from constant flow and constant pressure series were similar. Therefore, these studies indicate that lack of oxygen may induce vasodilation in small arteries.

Other studies further support the theory that oxygen lack dilates arterioles. Hyperoxia produced vasoconstriction in the canine hindlimb (Bachofen, 1971). The site of the constriction was the arterioles, as capillary and tissue P_{O_2} remained constant (Duling, 1972). Reich et al. (1970) studied the effect of breathing 100% oxygen at three atmospheres at rest and after exercise on calf blood flow in man. This procedure significantly reduced both resting and

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post-exercise flows. Hutchins et al. (1974) found that reducing air oxygen concentration to 18% produced a marked dilation only in terminal arterioles, metaarterioles, and distribution arterioles in rat muscle. All of these vessels were oriented transversely to the skeletal muscle fibers and had diameters of less than 40 μ. Pre-capillary sphincters and arterioles greater than 40 μ in diameter did not dilate. Duling and Berne (1970; 1971) compared intra- and extravascular oxygen tensions in arterioles and found them similar. Intravascular P_{O_2} fell progressively from the small arteries to the end of the terminal arterioles.

Venous oxygen changes during exercise in human leg muscles were recorded by Carlson and Pernow (1961). A few minutes after starting work venous oxygen saturation fell from resting levels of 53-81% to 22-42%. A slight but steady further decrease occurred with continued exercise. Venous oxygen saturation increased rapidly after exercise to values exceeding resting levels, reaching 70-89% saturation within five minutes. By 15 minutes post-exercise, venous oxygen saturation had returned to resting levels. Venous oxygen saturation never fell below 21%, even during heavy work. Venous lactate and pyruvate levels rose during exercise, indicating increased anaerobic metabolism by the tissue (Pernow and Wahren, 1962). Welch and Stainsby (1967), Greenwood et al. (1965), and Barcroft et al. (1963)

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confirmed these results, observing a relationship between contraction strength and amount of decline in venous oxygen saturation. Mohrman et al. (1973) and Mohrman and Sparks (1973) found that in the low constant-flow perfused canine calf muscle resistance changes returned to control with approximately the same time course as oxygen following a brief tetanus. Tominaga et al. (1973a) found a significant correlation between venous P_{O_2} and vascular resistance in the canine hindlimb; decreases in P_{O_2} were accompanied by decreases in vascular resistance.

Considerable evidence exists that oxygen cannot be the sole mediator of exercise hyperemia. Anrep and von Saalfield (1935) found that blood collected during exercise with restricted flow in the canine gastrocnemius still had potent vasodilator activity even after reoxygenation. In addition, venous blood collected during rhythmic contractions had a higher venous oxygen saturation than control venous blood. Love (1955) confirmed this latter result during sustained contractions in the human forearm. Morganroth et al. (1974) also found increased venous oxygen saturation in canine calf muscle during the entire period of exercise, which ranged from 20 to 60 minutes. Following exercise, oxygen saturation rapidly returned to control with a half-time that was five-fold faster than resistance. These experiments were performed at constant flow, with exercise induced by

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four twitches per second stimulation. Skinner and Costin (1971) reported that although hypoxemia produced vasodilation in the isolated gracilis muscle, the magnitude of the dilation was considerably less than that produced by exercise. Scott et al. (1965) found the canine forelimb relatively insensitive to changes in oxygen tension over the range of the usual arteriovenous difference.

Dornhorst and Whelan (1953) found that breathing 8% oxygen only slightly affected the recovery rate from exercise in humans. Likewise, Corcondilas et al. (1964) found that breathing oxygen had no effect on the dilation produced by brief contractions of varying strengths in the human forearm. Ross et al. (1964) compared increases in flow produced by exercise and by muscle perfusion with venous blood from a contracting donor muscle. An average flow increase of 173% was produced by exercise, with an average venous P_{O_2} of 25 mm Hg. Perfusion with venous blood increased flow by only 56%, with an average venous P_{O_2} of 23 mm Hg. Muscle exercise during venous blood perfusion was accompanied by an additional 143% flow increase, although venous P_{O_2} only decreased an additional 3%. Scott et al. (1970) reported that perfusion pressure decreased more during exercise than when the canine hindlimb was perfused with hypoxemic blood, although the venous P_{O_2} was kept higher in exercise by ventilation with 95% oxygen.

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Mohrman et al. (1973) found that with free-flow or high constant-flow perfusion the vascular response to a brief twitch returned to control before the decrease in end-capillary oxygen tension returned to control. Stowe (1974) corrected venous P_{O_2} from exercising canine muscle to the pre-exercise level, yet assay resistance still decreased to 71% of the exercise level.

Taken together, these studies indicate that oxygen may play some role in exercise hyperemia. However, that oxygen cannot be the complete cause appears equally evident.

Although the mechanism by which oxygen alters vascular smooth muscle tone is unknown, two theories have been proposed in this context. The first states that oxygen may directly alter vascular smooth muscle tone, since ATP is necessary for the contractile mechanism and adequate oxygen is necessary to maintain ATP stores. The second theory states that oxygen acts indirectly by determining the rate of release of some vasodilator substance. Perfusing isolated small arteries from muscle with bloods that had different oxygen contents produced vasodilation (Guyton et al., 1964). Since no extravascular tissues were present, it was suggested that decreases in oxygen directly alter vascular smooth muscle. Detar and Bohr (1968b) suggested that the vasodilation accompanying acute hypoxia in isolated arteries was a direct result of lack of sufficient oxygen to maintain

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ATP levels. After chronic hypoxia (6 to 8 hours) muscle tension development was much improved, suggesting development of anaerobic pathways for ATP synthesis (Detar and Bohr, 1968a). Hyperoxia-induced vasoconstriction in the canine hindlimb was not affected by wide variations in oxygen supply produced by different perfusion pressure rates of pure oxygen at three atmospheres (Bachofen, 1971). This again suggests a direct action of oxygen upon vascular smooth muscle, since the varying flows should have altered the concentration of any other vasodilator substance. Studies should be done to determine if the ATP content of vascular smooth muscle actually declines during hypoxia.

Other studies indicate that oxygen lack may produce vasodilation by release of some vasodilator substance. Honig (1968) suggested that oxygen sensitivity of vascular smooth muscle may be due to an inhibition of myosin ATPase by increases in inorganic phosphate and AMP during hypoxia. Berne et al. (1971) found increased total tissue levels of ADP, AMP, IMP, adenosine, inosine, and hypoxanthine during exercise in rat skeletal muscle. Dobson et al. (1971) found increased levels of adenosine in the venous effluent from the dog hindlimb during ischemic exercise. It was therefore suggested that hypoxia might induce the release of adenine compounds, which actually produced the dilation. However, Kontos et al. (1968a) found that dipyridamole did

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not affect the vascular response to hypoxia. This drug enhanced the vascular response to AMP and ATP. It should also be considered that if the AMP and/or adenosine produce dilation by production and accumulation within the vascular smooth muscle cells themselves, dipyridamole would not enhance the vascular response to hypoxia. Thus, the experiments of Kontos do not necessarily eliminate adenosine compounds as mediators of hypoxic dilation.

Several factors have been ruled out as mediators of hypoxic vasodilation. Scott et al. (1970) found that hypoxia alone did not cause increased venous potassium levels, thereby ruling this substance out as the mediator. Shepherd et al. (1973) found that varying the bathing solution P_{O_2} of isolated arteries from 680 to 0 mm Hg produced only small changes in arterial cAMP levels, suggesting that cAMP does not mediate oxygen's action on vessels. Hutchins et al. (1974) found that pharmacological blockade of sympathetic ganglia, α, β, or histaminergic receptors had no effect on the vascular response to mild systemic hypoxia, thereby tending to rule out nerves as mediators of hypoxic vasodilation.

Hydrogen Ion and Carbon Dioxide. Many experiments have been performed to determine whether or not carbon dioxide and hydrogen ions mediate exercise hyperemia. Bayliss (1900) found that increased carbon dioxide concentration

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produced vasodilation in Ringer perfused frog extremities. Lactic acid had a similar vasodilator potency. Diji (1959) confirmed the vasodilator action of carbon dioxide in the human hand. Kester et al. (1952), Deal and Green (1954), and Zsoter et al. (1961) found that large alterations in pH on either side of 7.4 produced considerable vasodilation in the canine hindlimb. Flows increased to 100% above controls. Duling (1973) reported an 18% increase in vessel diameter in the hamster cheek pouch as P_{CO_2} was increased from zero to 32 mm Hg. Crawford et al. (1959) decreased blood pH in dogs by subjecting them to 20% carbon dioxide for one hour. It was calculated that blood pH declined to 7.25, yet no increase in flow occurred, perhaps due to the sympatho-adrenal discharge which accompanies elevation of systemic P_{CO_2}.

Fleishman et al. (1957) studied the effects of small pH changes on vessel resistance. Blood pH was varied from 7.0 to 7.6 by hyperventilation and ventilation with 20% carbon dioxide. Small vessel resistance decreased with increases in hydrogen ions and increased with changes to the alkaline side. However, total resistance in the canine hindlimb did not change because of directionally opposite active changes in artery resistance. The large vessel response was related to nervous connections, as nerve block prevented it. Therefore, in intact limbs, small changes in

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Kontos et al. (1967) reported that human forearm resistance decreased 43% on increasing venous carbon dioxide tension from 40 to 48 mm Hg by intrabrachial infusion of a saline solution with a carbon dioxide tension greater than 600 mm Hg. In a further study, Kontos et al. (1968c) produced acidosis by a different means. Intraarterial infusion of acid phosphate buffer solutions produced a fall in human forearm venous blood pH from 7.34 to 7.24 and a rise in carbon dioxide tension from 42 to 52 mm Hg. This was accompanied by a 170% increase in forearm blood flow. Daugherty et al. (1971) locally reduced femoral blood pH to 7.19 by ventilation with 30% carbon dioxide and observed a fall in limb vascular resistance.

Radawski et al. (1972a) reported that at constant flow in the dog hindlimb increases in brachial artery P_{CO_2} from 17 to 137 mm Hg produced a decrease in skin small vessel resistance but had little effect on muscle. Emerson et al. (1974) demonstrated that large stepwise decreases in the pH of blood perfusing dog gracilis muscle produced by intra-arterial infusion of isotonic lactic acid solutions produced only small stepwise decreases in muscle vascular resistance. Molnar et al. (1962) obtained similar results. Therefore, although large changes in pH appear to alter total vascular

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Gollwitzer-Meier (1950) was one of the first to study venous pH changes during exercise in the canine gastrocnemius muscle. Three phases of pH change were observed: (1) an initial short phase of acidity; (2) a longer-lasting phase of alkalinity; and (3) a protracted, persistent phase of acidity. For example, during a ten second tetanus there was a small shift of pH to the acidic of short duration. Five to 20 seconds after the end of the contraction, at a time when venous outflow was reaching its maximum, the pH had returned to normal. A small pH shift to the alkaline then occurred, with a final return to resting values approximately one minute after the contraction. The hyperemia, however, continued for several minutes. The third phase of acidity was not apparent with such a brief tetanus. With longer periods of tetany or exercise, all three phases were observed. The second and third phases varied in duration and intensity depending on how strenuous the exercise was. The last phase of acidity was never associated with an increase in blood flow. In fact, flow had often returned to normal by this time. It was concluded that venous pH changes had no relationship to blood flow, either during or after exercise in the preparation used. Tominaga et al. (1973a) obtained results confirming this conclusion.

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Carlson and Pernow (1961) reported that venous pH declined from 7.41 to 7.25 during leg exercise at maximal load in humans. The venous pH was 7.24 five minutes after work. Scott et al. (1970) found a 270% increase in blood flow during sub-maximal exercise in the dog gastrocnemius muscle with only a 0.07 decline in venous pH. Ross et al. (1964) had obtained similar results. Radawski et al. (1972b) monitored pH changes during a two hour period of exercise in the isolated canine gracilis muscle. At two hours venous pH was only slightly below (7.30) control values (7.34). Stowe (1974) perfused the isolated, denervated canine gracilis muscle with blood pumped through an extracorporeal donor lung. The lung was ventilated with gas mixtures which decreased arterial blood pH. A pH decline from control levels of 7.41 to 7.06 produced only an 18% drop in vascular resistance. In addition, the increased vasodilator activity of the venous effluent during steady-state exercise was not abolished by correcting the pH. Kontos (1971) found that the alkaline buffer amine, tromethamine, only slightly reduced the dilation accompanying exercise in the human forearm. Also, a three-fold flow increase which accompanied forearm exercise was associated with only a 5 mm Hg increase in venous blood P_{CO_2}. Kontos et al. (1966) found that venous carbon dioxide tension would have had to rise by 19.4 mm Hg to account for the dilation accompanying five

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minutes of mild exercise in the human forearm. The actual rise was only 4.5 mm Hg. Gebert and Friedman (1973) showed that with rapid contractions, although tissue hydrogen ion activity increased, the increase was preceded by a transient decrease. Slow stimulation frequencies (less than one per minute) produced only a decrease in tissue hydrogen ion activity. Patients with McArdle's disease, suffering from a congenital absence of phosphorylase, showed no evidence of lactate formation or change of pH in working muscles, yet their functional hyperemia was as great as normal (Tobin and Coleman, 1965). Therefore, although carbon dioxide and hydrogen ions have been shown to be vasoactive locally, it is questionable as to whether their concentration changes during active hyperemia are of sufficient magnitude to be responsible for the dilation, either at onset of or during sustained exercise. However, it is possible that they may play a role in the dilation of maximal, severe exercise.

In one study on isolated bovine facial arteries Gebert et al. (1969) found that increased P_{CO_2} of the Tyrode bathing solution had no effect on the tone of the arteries if the pH was held constant. This indicates that increases in carbon dioxide may not dilate directly but via increases in hydrogen ions. However, Kontos et al. (1968b) found no significant difference between the vascular response to

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hypercapnia with acidosis and that to hypercapnia without acidosis in the human forearm. A direct effect of carbon dioxide was thus indicated. It was mentioned, however, that the pH inside the smooth muscle cells may have declined in spite of bicarbonate infusion, since carbon dioxide crosses cell membranes far more freely than bicarbonate. In this case hydrogen ions, not carbon dioxide, would be responsible for the dilation.

Several investigators have further studied the mechanism of action of changes in pH and P_{CO_2}. Kontos et al. (1970) found that alpha and beta adrenergic blockade did not prevent hypercapnic vasodilation. Fleishman et al. (1957) observed that the vascular response to hydrogen ions persisted in the absence of central nervous connections, circulating and locally released catecholamines, and following local parasympathetic blockade. Molnar et al. (1962) observed that venous potassium levels increased in association with acidosis, persumably by release from erythrocytes. Although the rise in potassium could explain part of the dilation which occurred, it was not of sufficient magnitude to entirely explain it. Also, nitric acid, which did not increase plasma potassium, produced dilation. Lade and Brown (1963) also observed that skeletal muscle lost potassium during hypercapnia induced by 30% carbon dioxide breathing in dogs. Contraction increased the rate of loss.

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Additional studies should be done to determine if efflux of adenine compounds increases with decreases in pH.

Osmolality. A large number of studies have shown that increased osmolality is associated with vasodilation. Marshall and Shepherd (1959) found that rapid local injections of two ml of 10-20% sodium chloride or 25-50% dextrose solutions into the canine femoral artery produced a prolonged two or three-fold increase in hindlimb blood flow. Continuous infusions of these substances for one minute caused flow increases which continued throughout the infusion. However, Gazitua et al. (1971) found that the initial dilation produced by hyperosmotic dextrose or sodium chloride in the canine forelimb waned during a ten minute infusion. Read et al. (1960) reported that rapid intravenous injections of 1500 mOsm/kg solutions produced peripheral vasodilation in dogs. Overbeck et al. (1961) demonstrated that locally increasing forelimb osmolality by 100 mOsm/kg decreased forelimb vascular resistance to 71% of the control when flow was held constant. Stainsby (1964) confirmed the vasodilator activity of various hyperosmotic solutions in the plasma-perfused gastrocnemius muscle. Stainsby and Fregly (1968) reported that decreases in resistance were proportional to increases in plasma osmolality. Large molecular weight substances such as dextran were as effective as smaller molecules in reducing resistance.

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In microscopic studies Gray (1971) localized hyperosmotic-induced vascular changes to the arterioles and precapillary vessels. Gray et al. (1968) found that hyperosmotic infusions of up to 40 mOsm/kg above control in cat skeletal muscle dilated precapillary sphincters but not veins. The hyperosmolarity also decreased considerably the arteriolar vascular response to sympathetic stimulation. However, Gebert et al. (1969) found that hyperosmotic sucrose or sodium chloride solutions had little effect on isolated bovine facial arteries.

Lundvall (1969) found that with hypertonic infusions to cat skeletal muscle, maximal dilation was reached when venous osmolality was raised by 40-100 mOsm/kg. In the human forearm osmolality increases of 15-20 mOsm/kg increased blood flow three to five times. Intramuscular deposits of Xe^{133} in resting muscle were cleared more rapidly when the tracer was dissolved in hypertonic than in isotonic solutions, indicating hyperemia when the hyperosmolality involved primarily the extravascular compartment (Lundvall et al., 1969). Overbeck and Grega (1970) showed that in humans intrabrachial infusions of hypertonic dextrose or sodium chloride increased cephalic venous osmolality 10 to 23 mOsm/kg and reduced forearm vascular resistance 4 to 14%. There was a positive linear correlation between the level of initial vascular resistance and magnitude of response to all hypertonic solutions. Stainsby and

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Barclay (1971) observed a 15-20 second lag between the start of a hyperosmotic infusion and the start of the dilation, indicating that hyperosmolality might not play a role in the initiation of active hyperemia. However, it was suggested that this delay might not apply to exercise, when the osmotically active substances could directly enter the interstitial space surrounding the resistance vessels. In addition, the delay may also have resulted from the dilution of the osmotically active particles at the initiation of their infusion.

Mellander et al. (1967) reported some of the earliest data on osmolality changes during exercise. Active hyperemia of five to 15 minutes duration in the lower leg muscles of the cat resulted in a sustained 80% drop in vascular resistance associated with a 38 mOsm/kg rise in effluent plasma osmolality. The increase in osmolality became more pronounced with time. Intraarterial infusion of hypertonic glucose or xylose solutions at rates producing similar changes in regional osmolarity produced a vascular response pattern similar to that of exercise. Lundvall et al. (1969) and Mellander and Lundvall (1971) found similar results in the human forearm during exercise. Lundvall (1972) found that tissue fluid hyperosmolality during exercise was largely due to increases in sodium and lactate concentrations, based on analyses of venous plasma.

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Scott et al. (1970) obtained different results. Although venous osmolality increased during the first minute of exercise in the canine hindlimb, it had returned to normal by the fifth minute whereas blood flow increased further. In addition, comparable increases in venous osmolality by infusions failed to produce similar increases in blood flow. Much larger increases still failed to reproduce exercise hyperemia. Scott and Radawski (1971) confirmed these results. They also observed a greater increase in venous osmolality with exercise during constant flow than during natural flow. Tominaga et al. (1973a) found that although venous osmolality remained elevated during eight minutes of exercise in the dog hindlimb, there was no correlation with vascular resistance changes. Osmolality soon returned to control levels following exercise. Hilton and Hudlicka (1971) found no relationship between exercise and venous osmolality in the cat gastrocnemius. Lundvall (1972) found a decline in venous osmolality during prolonged, strenuous work. Radawski et al. (1972b) found no arteriovenous difference for osmolality after two hours of exercise in the canine gracilis muscle. Morganroth et al. (1974) found similar results after 20 and 60 minute periods of exercise. During recovery, venous osmolality was actually below control levels. Murray et al. (1974) also obtained similar results. In addition, they studied venous osmolality

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changes in response to a one second tetanus, finding only a 1 mOsm/l increase for a 30% fall in resistance. It was thus concluded that changes in osmolality were too small to account for the dilation following brief tetanus or the sustained dilation of long exercise and in the wrong direction to account for post-exercise hyperemia. The possibility that venous osmolality may be less than tissue osmolality during exercise should also be considered, since outflow osmolality from the tissue may be diluted by non-nutritional flow. It was suggested that hyperosmolality might play a role in the first several minutes of exercise hyperemia. Lundvall (1972) reported that during light work in cats 40% of the hyperemia could be attributed to hyperosmolality; during heavy, short-term exercise 60% of the dilation could be attributed to hyperosmolality.

The mechanism by which hyperosmolality induces vasodilation has been investigated by Arvill et al. (1969). A relation between cell volume and activity in vascular smooth muscle was found. Intracellular fluid volume of rat portal vein strips decreased 34% after 20 minutes of exposure to Krebs solution plus 150 mmoles/l of sucrose. During this time spontaneous muscle activity was inhibited. Immediate excitation was produced on return to a normal isoosmotic medium. Mellander et al. (1967) and Mellander and Lundvall (1971) found that hyperosmolality exerted negative

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chronotropic, dromotropic, and inotropic effects on vascular smooth muscle activity of the portal vein. A doubling of the potassium concentration of the hyperosmotic solution restored smooth muscle activity to a pattern similar to controls. It was therefore suggested that hyperosmolality might actively dilate by increasing the intracellular potassium concentration subsequent to osmotic withdrawal of water from the cells, thereby producing hyperpolarization and relaxation. Other ion fluxes could also change.

Adenine Compounds. The potent vasodilator capacity of the adenine compounds has been known since the studies of Drury and Szent-Gyorgyi (1929). Folkow et al. (1948) found that intraarterial injection of 0.1-0.2 γ of ATP into cat hindlimbs produced considerable vasodilation. Injection of 2 γ doubled the rate of flow. The threshold dose for the effect was 0.05-0.1 γ, a potency about one-fifth to one-fifteenth that of acetylcholine. Sydow and Ahlquist (1950) demonstrated the vasodilator effect of AMP was more prolonged than that of adenosine in the dog. Duff et al. (1954) found a three-fold increase in muscle blood flow after infusion of 16 μg ATP/min. into the human forearm. Dobson et al. (1971) found that the arterial blood levels required to elicit detectable vasodilation for ATP, ADP and AMP were 28×10^{-8} M, 27×10^{-8} M, and 31×10^{-8} M, respectively. Mg-ATP was a more powerful vasodilator than

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Na-ATP (Duff et al., 1954). Frohlich (1963) localized the vasodilator effect of the three adenine nucleotides to small vessels, particularly the arterioles, in the dog. Kjellmer and Odelram (1965) found dilation of both arterial and venous vessels with ATP infusion in cats. The ATP caused no change in capillary permeability. ATP and ADP were four times as potent as AMP and adenosine in increasing coronary blood flow (Wolf and Berne, 1956). In addition to vasodilation, Raberger et al. (1973) found that adenosine infusion into the canine hindlimb was associated with reduced oxygen consumption, increased carbon dioxide production, increased uptake of glucose, glycerol, and ketone bodies, and increased release of free fatty acids and lactate.

Forrester (1966) provided the first direct evidence that ATP may be involved in active hyperemia. He found ATP released from active frog skeletal muscle in vitro. Further studies by Boyd and Forrester (1968) demonstrated that the ATP did not come from muscle cell damage, as the potassium ion level in the plasma did not increase. The mean output of ATP by the muscles for a 30 minute period of stimulation was 8 µg/gm muscle, amounting to a loss of about 1-2% of the intracellular ATP. Forrester and Lind (1969), using the firefly extract analysis for ATP, identified ATP in human plasma, both in resting and exercising subjects.

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Venous ATP levels of exercising subjects rose consistently above resting values, indicating addition of ATP to blood passing through the muscle bed. The mean plasma ATP concentration was 500 ng/ml at rest. The pattern of forearm blood flow and the ATP output were similar, increasing during contraction, rising to a peak value immediately after the contraction, and subsiding slowly to control values. Forrester (1972) found that with a more refined technique no ATP could be identified in the venous effluent plasma from an occluded forearm without exercise. In the venous effluent from occluded, exercising muscle, 0.033 to 1.0 nmole ATP/ml of plasma was observed, amounting to 1.5 μg ATP/gm wet weight of forearm muscle. After taking into account the amount of ATP lost by degradation, it was concluded that ATP was released in sufficient quantities (15 μg/min.) to mediate active hyperemia. Forrester and Hamilton (1975) found increased levels of ATP in the venous plasma from exercising cat soleus muscle. No significant increase in ATP occurred when the muscle was artificially perfused. In this latter case control ATP levels were considerably higher than in the blood perfused muscle, the difference being 0.85 μM.

Chen et al. (1972b) also found ATP in the venous effluent during active hyperemia in canine skeletal muscle. Control ATP levels were 206 ng/ml plasma in the femoral

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artery and 165 ng/ml in the femoral vein. During active hyperemia, femoral venous ATP levels rose to approximately 450 ng/ml plasma. Femoral venous AMP levels also increased from 52 to 188 ng/ml plasma. Increased flow correlated with increased ATP levels. Post-tetany ATP and AMP levels were still considerably elevated above control levels.

Parkinson (1973) found that levels of all three adenine nucleotides rose in the venous plasma following a rowing exercise in humans. The rise was proportionate to the severity of the exercise. AMP appeared only during severe exercise. ATP and ADP rose to approximately 500 ng/ml and AMP to approximately 300 ng/ml during severe exercise. Abood et al. (1962) reported efflux of ATP from frog muscle during depolarization.

Scott et al. (1965) found that the blood from the exercising canine hindlimb dilated the forelimb but constricted the kidney vasculature. They further demonstrated that the same responses could be produced by injection of adenosine and AMP but not ATP or other substances. With two kidneys in series ATP injected into the left renal artery dilated the left kidney but constricted the right kidney, indicating a rapid breakdown of ATP or a release of a vasoconstrictor from the donor kidney. Scott et al. (1971), in further bioassay studies in which lag time was considerably decreased and sensitivity increased, found that hindlimb

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blood during nerve stimulation at 6 cps or tetany produced a fall in kidney resistance. On stopping tetany kidney resistance rose. Stimulation plus occlusion in the hindlimb constricted the kidney. Therefore, these studies indicate that ATP or ADP may indeed be released during active hyperemia in skeletal muscle but also that their degradation in the blood is probably very rapid.

Brashear et al. (1968) found no detectable increases in venous plasma ATP or ADP following five minutes of treadmill exercise in humans. However, the sensitivity of their assay was only 0.2 mg%, and they used only cold to prevent the rapid breakdown of these compounds which occurs in whole blood and plasma (Collingsworth et al., 1974). Hilton (1962) found no ATP released during contractions in cat gastrocnemius muscle. This was true both of venous effluent and tissue fluid. The fluorometric assay used was sensitive enough to detect 100 ng/ml of ATP. ATP breakdown between time of collection and time of assay may have been responsible for these negative results. Kjellmer and Odelram (1965) suggested that since ATP dilates both arteries and veins, it would be unlikely to mediate exercise hyperemia involving the whole organism, where arteriolar dilation and venous constriction occur due to sympathoadrenal discharge. However, Nagle et al. (1968) showed that local venous resistance decreases with active hyperemia.

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Dobson et al. (1971) detected no adenine nucleotides in the venous plasma following five minutes of ischemic contractions in the dog hindlimb. However, no adequate measures were used to prevent adenine nucleotide breakdown during the isolation of the plasma. No ATP was detected in the bathing medium of the isolated frog sartorius muscle after 30 minutes of contraction; the assay used was sensitive enough to detect 1×10^{-8} M ATP concentrations. Bockman et al. (1975b) found no increase in venous plasma ATP during or following tetanic contraction in dog or rat hindlimbs. Therefore, since we have both negative and positive findings, it is obviously controversial as to whether or not adenine nucleotides mediate active hyperemia.

Considerable evidence exists indicating that adenosine may mediate blood flow changes in skeletal muscle and several other tissues. Jacob and Berne (1961) proposed that adenosine may be the mediator of coronary vasodilation in the hypoxic heart. This proposal is based on the observation that inosine and hypoxanthine were present in the perfusate and coronary sinus blood of severely hypoxic hearts. Due to the rapid breakdown of adenosine to these products in the body, it was suggested that adenosine was the substance released by hypoxic myocardial cells, with resultant vasodilation. Berne (1963) found that 3 to 27 times more moles of inosine and hypoxanthine were released

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from the hypoxic heart than were required to double coronary flow when infused as moles of adenosine into the left coronary artery. These substances were not detected in the arterial blood or in venous blood from a well-oxygenated heart. Therefore, Berne proposed that during hypoxia the decreased oxygen tension caused breakdown of heart muscle adenine nucleotides to adenosine. The adenosine would then diffuse out of the cells and reach the coronary arteries via the interstitial fluid, with resultant dilation. The increased coronary blood flow which would occur would raise the oxygen tension, thereby reducing the rate of degradation of adenine nucleotides to adenosine. This feedback mechanism would thus provide a way to adjust coronary flow to meet the heart's oxygen needs.

In support of Berne's theory Imai et al. (1964) found adenosine in underperfused rabbit myocardium under conditions of complete ischemia. Rubio and Berne (1969) found adenosine released by the normal myocardium continuously into the surrounding interstitial fluid (pericardial fluid). The normal adenosine concentration of the pericardial fluid was approximately 10.9×10^{-7} M, presumably a basal level. These results indicate that interstitial fluid adenosine levels could regulate coronary blood flow to maintain a proper oxygen balance.

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Dobson et al. (1971) found adenosine to increase five-fold in the venous plasma following ischemic muscle contraction. Venous inosine and hypoxanthine increased 22 and 270-fold, respectively. Tissue adenosine levels increased from 0.7 nmole/gm to 1.5 nmole/gm in ischemic, contracting muscle. Berne et al. (1971) found large increments in tissue IMP and small increases in tissue adenosine during ischemic skeletal muscle contractions in the rat. Bockman et al. (1975a) found muscle adenosine levels elevated after 10 and 25 minutes of contraction in the dog hindlimb but not after five minutes of contraction. Thirty minutes after contraction tissue adenosine content was slightly below control levels. No increase in the venous plasma adenosine levels occurred during the contractions. Bockman et al. (1975b) found venous adenosine levels increased during contraction in artificially perfused rat hindlimbs.

Tominaga et al. (1973d) also found increased levels of venous plasma adenosine and/or AMP during contractions in the dog hindlimb. Levels rose from a control of 259 nmoles/min./100 gm to 1030 during contractions. An increase also followed ischemic contractions. Watanabe et al. (1973) found that the intracellular changes in ammonia, lactate, inorganic phosphate, potassium, and oxygen which occur during exercise depressed adenosine and AMP aminohydrolases. In this way AMP or adenosine could accumulate during

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exercise and produce dilation. Tominaga et al. (1973c) found that recovery of injected ^{14}C-adenosine decreased during contractions in the dog hindlimb, indicating increased uptake of the ^{14}C during exercise. However, adenosine concentration remained constant in the venous plasma during exercise. Therefore, increased release of adenosine must have occurred to maintain a constant concentration. Tominaga et al. (1975) found increases in AMP and/or adenosine with contractions and ischemic contractions under constant flow but not with constant pressure.

Recently, studies have been done on the role of adenosine in the regulation of kidney and brain blood flow. Thomas et al. (1975) found no correlation between tissue adenosine levels and renal artery perfusion pressure. Berne et al. (1974) demonstrated that topical application of adenosine to cat pial arterioles produced dilation roughly proportional to the dose used. However, intra-arterially administered adenosine did not appear to cross the blood-brain barrier. In further studies Rubio et al. (1975) found that brain hypoxia produced by electrical stimulation of the cortex, decreased arterial perfusion pressure, or ventilation with 5% oxygen all increased tissue adenosine levels. It was suggested that adenosine and hydrogen ions may act synergistically to regulate cerebral blood flow.

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Three possibilities exist for the source of the observed increases in adenine compounds: nerve, muscle, and blood cells. Early studies indicated that extracellular adenosine could not have come from skeletal muscle, since the predominant pathway for AMP breakdown in skeletal muscle is via AMP aminohydrolase to IMP (Imai et al., 1964). In addition, adenosine deaminase levels are high within muscle cells, resulting in low intracellular adenosine levels. Rubio et al. (1973) localized skeletal muscle 5'-nucleotidase activity by a histochemical technique to endothelium and to localized zones within muscle cells in close proximity to blood vessels in rats and guinea pigs. This enzyme converts AMP to adenosine. Therefore, this study showed that skeletal muscle adenosine is produced only in the vicinity of the blood vessels, where it could reach a concentration high enough to produce vasodilation.*

Other studies indicate that adenine nucleotides may pass through muscle cell membranes and thus could possibly become chemical messengers. Chaudry and Gould (1970) reported evidence for the direct entry of ATP and ADP into the rat soleus muscle. More ^{14}C-ATP was found in the cells when ^{14}C-ATP or ^{14}C-ADP were added to the incubation medium

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*For a more complete discussion of adenine compound metabolism in skeletal muscle, see pp. 56-60, Collingsworth, A. H., M. S. Thesis, 1973.

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than when ^{14}C-adenosine was added to the medium. Chaudry et al. (1975b) further confirmed this finding. Abood et al. (1962) found efflux of ^{32}P-labelled ATP during muscle cell depolarization. Boyd and Forrester (1968) suggested that ATP may leave muscles by way of the transverse tubular system of the sarcoplasmic reticulum rather than by passing directly through the membrane.

Blood cells may have been the source of the adenine nucleotides observed in some studies. Forrester (1972) found that control levels of ATP were eliminated by switching from EDTA to citrate as anticoagulant. EDTA has been shown to cause adenine nucleotide efflux from cells (Abood et al., 1962; Bockman et al., 1975b; Collingsworth and Liu, unpublished). Damaged platelets could also release considerable quantities of ATP and ADP (Holmsen, 1967).

Considerable evidence has accumulated that nerves may be a source of extracellular ATP.* Burnstock (1972) has published an excellent review of this area. Hilton (1953) suggested that post-exercise hyperemia was due to a local axon reflex with an unknown chemical transmitter, since cocaine abolished the response. Honig and Frierson (1974) attributed post-exercise hyperemia to intraarterial ganglion cells, since procaine partially blocked the response.

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*For a more complete discussion of this subject see pp. 29-35, Collingsworth, A. H., M. S. Thesis, 1973.

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Again, a transmitter was not identified. Holton (1959) found ATP released into the venous effluent upon antidromic stimulation of the great auricular nerve or the skin in the rabbit. The ATP levels observed could not be accounted for by erythrocyte hemolysis. Degeneration of the nerve prevented the ATP efflux upon stimulation of the skin. Ballard et al. (1970) demonstrated non-cholinergic vasodilator nerves in the canine paw. It was suggested that one of the adenine compounds might be the transmitter substance.

Su (1975) found ^{3}H-adenosine taken up by the isolated thoracic aorta, ear artery and portal vein of the rabbit. It was transformed mainly to ^{3}H-ATP within the cells. On transmural stimulation tritiated adenosine and inosine accounted for 80% of the total released ^{3}H. Adenine nucleotides made up the rest. Tetrodotoxin abolished the response. Phenoxybenzamine prevented contraction but did not prevent ^{3}H efflux. Kuperman et al. (1964) found that electrical stimulation of the frog sciatic nerve resulted in a considerable increase in labelled AMP and ATP efflux. Increased calcium efflux also occurred during stimulation, suggesting a possible binding or interaction of the two substances.

In a study on the rat phrenic nerve-hemidiaphragm preparation, Silinsky and Hubbard (1973) and Silinsky (1975) reported the release of ATP from motor nerve terminals.

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No stimulus-specific release of purines was found in a normal preparation. However, in a highly curarized preparation, approximately six times as much ADP was found in the bath after stimulation as was found there in the control period. Since no ATP was found, Silinsky suspected ATPase was present, and therefore a perfusate consisting of magnesium-free saline with 0.1 mM calcium added was used to minimize ATPase activity. In these curarized preparations ATP levels in the bath increased to approximately four times control levels upon nerve stimulation and depolarization induced by a hyperosmotic bathing medium. It was suggested that the ATP was released from the motor nerve terminals, since acetylcholine release paralleled ATP release. However, the fact that a hyperosmotic solution might in some way affect the permeability of the muscle membrane and thereby result in the efflux of ATP was not considered. Some further evidence was presented in favor of the nerve terminal source of the ATP, however. When no calcium was present in the hyperosmotic bathing medium (a condition which could prevent acetylcholine release upon nerve stimulation), no ATP efflux occurred on nerve stimulation. It was suggested that the ATP might function in complexing acetylcholine in nerve storage vesicles.

In a study to extend Silinsky's 1973 results, however, Kato et al. (1974) were unable to find ATP released upon

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stimulation of cat superior cervical ganglion. This preparation avoided any artifacts due to the presence of muscle. Stimulation was produced electrically or with a high potassium perfusate. Although acetylcholine was released by both types of stimulation, ATP release could not be detected during either type. However, the fact that a different type of nerve preparation was used must be considered as a possible source of the negative results. ATP release was found immediately following a change in the perfusate from normal Ringer's to one lacking calcium and magnesium but containing barium and EDTA. This modified perfusate resulted in a transient depolarization of post-ganglionic fibers and a contraction of the nicitating membrane. However, no concurrent release of acetylcholine was found, and the source of the ATP was not determined.

The mechanism by which adenine compounds dilate is as yet largely unknown. Folkow (1949b) reported that atropine and neoantergan (antihistamine) did not block ATP-induced vasodilation. Denervation also had no effect. Weissel et al. (1973) suggested that the dilator action of adenosine may be coupled in some way to its metabolic effects. Needleman et al. (1974) found that ATP and ADP stimulated prostaglandin release from many tissues. This would indicate an indirect dilator effect of adenine compounds, with prostaglandins actually accomplishing the dilation.

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Adenine compounds could directly dilate by decreasing membrane permeability to calcium. They could also stimulate a coupled sodium-potassium pump, decrease membrane permeability to sodium, or increase membrane permeability to potassium. Any of these effects would hyperpolarize and result in relaxation. Much more work needs to be done to determine the actual mechanism by which adenine compounds dilate.

Other Dilator Substances. A variety of substances other than those already discussed have been suggested as mediators of local blood flow regulation. Histamine was early implicated in such a role. Anrep and Barsoum (1935) found increased histamine levels in the venous blood during exercise in dogs. The stronger the contractions, the more histamine appeared. Stimulation of vasodilator or vasoconstrictor nerves alone did not cause an increase in venous histamine. Anrep et al. (1939) confirmed these results. They also showed that pre-existing muscle histamine stores were depleted during contractions of prolonged duration. Increased histamine was found in the venous effluent from the exercising forearm by Anrep et al. (1944). Graham et al. (1964) found that exercise and catecholamines caused increased histamine forming capacity in mouse muscle. Removal of the adrenal medulla prevented the rise in histamine forming capacity caused by exercise. Schayer (1965)

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reported that histamine is continually synthesized within smooth muscle cells and that its rate of synthesis can be adjusted to meet various conditions. Antihistamines caused contraction of microvessels in the rat (Altura and Zweifach, 1965).

However, Emmelin et al. (1941) found no increase in venous histamine during tetanization of the canine gastrocnemius. Altura and Zweifach (1967) found that intrinsic histamine formation was not regularly associated with dilation in the rat. Also, histamine was shown to be a rather non-specific dilator of the vasculature (Altura and Zweifach, 1965). Therefore, it is still controversial as to what role histamine might play in exercise hyperemia.

Fox et al. (1961) demonstrated that bradykinin is a potent vasodilator in the human forearm. Allwood and Lewis (1964) found no increase in venous bradykinin following exercise in the human forearm. However, the substance could not be excluded as a mediator of the hyperemia, since large increases in forearm blood flow occurred upon intra-arterial bradykinin infusion without a detectable venous increase in bradykinin. Carretero et al. (1965) also found no increase in venous bradykinin following exercise in humans. More conclusively, Webster et al. (1967) showed that carboxypeptidase B, an inhibitor of bradykinin-induced vasodilation, had no effect on the vasodilation produced by muscle contraction in the canine gracilis muscle.

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Barcroft et al. (1970) found evidence regarding inorganic phosphate in humans. During sustained or rhythmic exercise venous plasma phosphate increased by 20%, while blood flow increased ten times. Infusion of phosphate until a four-fold increase had been produced changed blood flow very little. However, in the cat phosphate was shown to dilate adequately enough to account for the hyperemia produced by a short tetany (Hilton and Vrbova, 1970; Hilton and Chir, 1971). Hilton and Hudlicka (1971) found that in the cat release of inorganic phosphate closely paralleled increases in blood flow associated with exercise. Dobson et al. (1971) found no increase in phosphate release from the artificially perfused, exercising frog sartorius muscle or the exercising canine hindlimb. Tominaga et al. (1973a) found no initial increase in phosphate release during exercise in the dog hindlimb. There was a gradual increase after stabilization of the vascular resistance, but the increase in phosphate did not correlate with the increase in flow. Therefore, the role of phosphate in active hyperemia is controversial and appears to depend on the species studies.

Haddy (1960) reported that local magnesium excess produced arteriolar dilation in the dog forelimb. Whang and Wagner (1966), following forearm exercise in humans, found an increase in venous magnesium, presumably due to a

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decrease in the amount of water present. Scott et al. (1970) reported that the magnesium increase was only 5% greater than the control value and had disappeared by the fifth minute of gracilis exercise with natural flow. Radawski et al. (1972b) found no arterio-venous magnesium difference after two hours of exercise in the canine gracilis muscle.

Hedwall et al. (1971) showed that the prostaglandin PGE_1 was a potent vasodilator in the dog gracilis muscle and hindpaw. The substance also had some inhibitory effect on vasoconstrictor agents such as nerve stimulation and norepinephrine. Daugherty (1971) found that PGE_1 dilated both pre- and post-capillary vessels in the dog forelimb. However, no studies were found regarding a specific role of prostaglandins in active hyperemia.

Acetate has been shown to produce arteriolar dilation and venous constriction (Overbeck et al., 1961) but no studies were available on its role in active hyperemia. Frolich (1965) specifically tested the vascular effects of seven intermediary products of oxidative metabolism in the dog forelimb. The metabolites were acetate, citrate, fumarate, malate, α-ketoglutarate, oxaloacetate, and succinate. Submaximal doses of each substance (2.47 μmole/min.) produced prompt vasodilation which was localized to the arterioles. Venous lactate and pyruvate increased during and after leg exercise in humans (Carlson and Pernow, 1961).

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Lactate remained elevated for a 20 minute period of exercise in dog calf muscles but declined to control levels within a 60 minute period of exercise (Morganroth et al., 1974). Thus, although these substances produce dilation, their degree of change during alterations in blood flow is controversial and in some cases unknown.

Interaction of Substances. Relatively few studies have been done to determine the combined effects of factors during active hyperemia. Billings and Maegraith (1937) were among the first to suggest that an accumulation of substances might interact to produce vasodilation. Kontos et al. (1966) reported that the decrease in venous blood oxygen tension and the increase in venous blood carbon dioxide tension during five minutes of mild exercise in the human forearm were too small to entirely account for the increased blood flow. Kontos et al. (1970) found in the human forearm that combined hypoxia and hypercapnia equal in magnitude to that observed during ischemia of five minutes duration resulted in vasodilation averaging 64% of that produced by the ischemia. The combined effect was not substantially greater than the additive effects of hypoxia alone and hypercapnia alone.

Skinner (1967) found that perfusion of the isolated canine gracilis muscle with blood low in oxygen and high in potasssium produced greater dilation than perfusion with

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blood low in both oxygen and potassium. Likewise, the constrictor response to sympathetic stimulation was reduced more by perfusion with blood low in oxygen and high in potassium than by perfusion with blood low in both substances. Skinner and Powell (1967) demonstrated in the canine gracilis muscle that increases in venous potassium were never associated with maximal dilation as long as oxygen levels remained normal. When perfusing with oxygen deficient blood, the higher the potassium concentration, the greater and more rapid was the decrease in vascular resistance. In further studies Skinner and Costin (1970) and Skinner and Costin (1971) found that blood with decreased oxygen and increased osmolality was a more potent dilator than oxygen deficient blood alone, both before and during sympathetic stimulation. Perfusion with oxygen deficient, hyperkalemic, hyperosmotic blood produced greater dilation both before and during sympathetic stimulation than perfusion with oxygen deficient and hyperkalemic blood.

Stowe et al. (1974) found that reduction of both P_{O_2} and pH caused a greater drop in canine gracilis muscle resistance than either alone. A 40% drop in resistance was produced by reducing both P_{O_2} and pH to levels found during heavy exercise (23 and 7.10). Mild exercise produced only moderate decreases in P_{O_2} and pH (34 and 7.28) but a 38% decrease in resistance. Stowe (1974) reported that the

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enhanced vasodilator activity of venous blood during steady state exercise can be completely abolished by returning pH and P_{O_2} to pre-exercise levels. These two parameters remained decreased throughout sustained exercise. No correction of the effluent for potassium or osmolality was necessary.

Honig (1968) found that phosphate and AMP together produce greater inhibition of ATPase activity than the sum of the separate effects of the two substances. Since intracellular levels of these metabolites may rise with oxygen lack, a mechanism may exist for chemical feedback on the contractile activity of smooth muscle cells. Concentrations of phosphate and AMP equivalent to those found in ischemic muscle almost completely inhibited contraction-coupled ATP hydrolysis.

2. Reactive Hyperemia of Skeletal Muscle

Two major theories have been proposed to explain the vasodilation following arterial occlusion first observed by Conheim in 1872. The metabolic theory states that chemicals build up during the occlusion and produce the dilation. Freeburg and Hyman (1960) found a 30-60 second delay in vasodilation following arterial occlusion in the human leg and a second flow maximum 30 to 45 seconds later, suggesting a metabolic cause for the dilation. A stable metabolic

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dilator could recirculate, producing the second maximum. The finding that prolongation of the occlusion caused a proportionate increase in excess blood flow also supports this theory (Patterson and Whelan, 1955). However, their additional finding that there is great variability in the amount of blood flow repayment under the same conditions does not support this theory as well. In most cases the debt was considerably overpaid. The myogenic theory holds that the decreased blood pressure during the occlusion produces the dilation. A third possibility is that both factors are involved in mediating reactive hyperemia.

Early studies focused on the vasodilator histamine as the mediator of reactive hyperemia. Lewis (1927) was the first to suggest this possibility. Anrep et al. (1944) found increased plasma levels of histamine in human venous blood following arterial occlusion of 10 or 20 minutes duration. Restriction of venous outflow by compression greatly enhanced the amount of the increase. With this method histamine levels were elevated for two minutes following the occlusion. With a free circulation histamine levels returned to normal long before the end of the hyperemia. Billings and Maegraith (1937) found histamine in the venous blood from ischemic rabbit tissues. Kwiatkowski (1941), however, found no increase in venous histamine levels in man or rabbits following release of arterial

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occlusion. In addition, a cysteine block of histamine did not alter the hyperemic response. Emmelin et al. (1941) confirmed these negative findings in both humans and dogs. These results were attributed by others (Anrep et al., 1944) to insufficient control of venous flow and collection of samples at a time when histamine levels had already declined.

Folkow et al. (1948), using more refined techniques, also found no evidence that histamine was a mediator of reactive hyperemia. Reactive hyperemia was of the same magnitude whether an animal's blood vessels were very sensitive to histamine or relatively insensitive. Rendering the blood vessels completely insensitive to histamine before arterial occlusion also had no effect on the magnitude of the reactive hyperemia. Administration of benadryl or neoantergan, antihistamine agents, did not alter the hyperemia. Duff et al. (1955) found that hyperemia following prolonged arterial occlusion (longer than ten minutes) was indeed reduced by antihistamines. It was therefore suggested that histamine may partially mediate reactive hyperemia following prolonged arrest of the circulation. Considering all the evidence, histamine does not appear to be a good candidate for mediating reactive hyperemia.

Horton (1964) examined venous blood collected during reactive hyperemia in dog hindlimbs for increases in kinins. Although no plasma kinins were detected in this blood,

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serotonin increased seven-fold above control levels. However, this substance was not implicated as a mediator of hyperemia, since its intraarterial infusion did not alter blood flow. Its increase was instead attributed to an anti-clotting function. Allwood and Lewis (1964) obtained similar results. Webster et al. (1967) also found no evidence for kinin participation in reactive hyperemia. The kinin blocker carboxypeptidase B had no effect on the hyperemic response to occlusion.

Oxygen is another substance which could mediate reactive hyperemia. Arterial occlusion would lead to a decrease in tissue oxygen levels. Dornhorst and Whelan (1953) found that reduced arterial oxygen saturation both before and after circulatory arrest had no effect on the duration of the subsequent reactive hyperemia. In addition, McNeill (1956) measured venous oxygen saturation during reactive hyperemia and found it to be decreased only during the early stage of the hyperemia. Control oxygen levels were reached considerably before the end of the vasodilation. It was thus considered unlikely that tissue hypoxia existed during the entire hyperemic period and that oxygen deficit mediated reactive hyperemia. Kontos et al. (1965) confirmed these results. Kontos et al. (1970) found that for equal decreases in deep forearm venous blood P_{O_2}, the vasodilator response to hypoxia averaged only 26% of that produced by ischemia.

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Also, the hyperemia following ischemia was not modified by breathing 100% oxygen, thereby maintaining deep venous blood P_{O_2} at a level above that seen with free circulation during room air breathing.

In support of oxygen as a mediator of reactive hyperemia, Crawford et al. (1959) showed that blood flow in the canine hindlimb increased nearly proportional with the degree of hypoxia. More conclusively, Fairchild et al. (1966) occluded arterial flow to the canine hindlimb for three to ten minutes. On release of the occlusion, the limbs were perfused with deoxygenated blood. No recovery from the reactive hyperemia occurred until the perfusion blood was reoxygenated ten minutes later. Following reoxygenation recovery occurred within a few minutes. Whether the oxygen lack directly caused the dilation or only did so via release of a mediator was not answered in these experiments. It was suggested that washout of metabolites was not a major cause of reactive hyperemia. Tominaga et al. (1973a) also showed that venous P_{O_2} correlated with the period of reactive hyperemia following a two minute occlusion.

Baetjer (1935), working with cat hindlimbs, found increases in venous blood potassium following reduction of the arterial inflow by 20% or more. However, Scott and Radawski (1971) measured control and hyperemic levels of

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osmolality, magnesium, and potassium in the venous blood from the canine gracilis muscle. Following a five minute arterial occlusion, no changes in any of these substances occurred in the venous outflow. Rudko and Haddy (1965) and Tominaga et al. (1973a) obtained similar results.

Billings and Maegraith (1937) were among the first to report that tissues become acid upon occlusion of their blood supply. Kontos et al. (1965) measured venous pH and CO_2 levels in venous blood during rest and reactive hyperemia brought about by 30 second or larger arterial occlusions in the canine hindlimb. Venous P_{CO_2} increased rapidly and then declined gradually to control levels, following blood flow with a delay of several seconds. Venous pH changes were inversely proportional to those of P_{CO_2}. Hypocapnia produced by hyperventilation decreased the hyperemic response to a 30 second occlusion; hypercapnia produced by acetazolamide administration increased the hyperemic response. Scott et al. (1970) found that pH fell from 7.38 to 7.28 during reactive hyperemia in the canine hindlimb. Kontos and Patterson (1964) found similar results in the human forearm. Flow during reactive hyperemia increased ten times the control value; venous pH fell by only 0.03 units and P_{CO_2} rose 10 mm Hg. In this study they found also that intravenous infusion of sodium bicarbonate had no effect on reactive hyperemia, indicating mediation

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of the hyperemia via carbon dioxide rather than pH. Kontos et al. (1970) observed greater increases in blood flow after circulatory arrest in the human forearm during carbon dioxide breathing then during room air breathing. Flow increases after ischemia were maintained until carbon dioxide administration was stopped. For equal increases in deep forearm venous blood P_{CO_2}, the vasodilator response to hypercapnia averaged 60% of that following ischemia. Kontos et al. (1971) confirmed this latter result using acid infusion in the canine gastrocnemius muscle. Kontos (1971) found that the alkaline buffer amine, tromethamine, markedly reduced the vasodilator response to ischemia in the dog and in humans. However, Tominaga et al. (1973a) found no correlation between venous pH or P_{CO_2} and vascular resistance following a two minute arterial occlusion. Therefore, whether or not the accumulation of carbon dioxide in ischemic tissues may act as a mediator of reactive hyperamia is still controversial.

The adenine compounds have been suggested as mediators of reactive hyperemia. Gordon (1962) found that AMP levels in the venous plasma from the rabbit kidney following arterial occlusion rose to 30 µg/ml. However, varying the duration of the occlusion from three to 30 minutes did not change the level of AMP found. No AMP was detected in venous blood from the rabbit hindlimb following a 30 minute

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arterial occlusion. Berne et al. (1963) and Imai et al. (1964) likewise found no increase in adenosine and only slight increases in AMP in ischemic rabbit skeletal muscle. IMP levels increased. ATP levels decreased in rabbit muscle as early as four hours following ligation of the iliac and femoral arteries (Grigor'eva et al., 1965). Interruption of the coronary circulation increased cardiac tissue adenosine levels. In further studies of this type (Berne et al., 1971) the venous blood from contracting, ischemic canine skeletal muscle was analyzed for adenine nucleotides and adenosine. Only the adenosine levels rose above control values, from 0.03 nmoles/ml to 0.12 nmoles/ml. Dobson (1971) confirmed these results and also found that the muscle adenosine content rose from a control level of 0.7 to 1.5 nmole/gm during ischemic contraction. Forrester (1972) found no ATP in the venous plasma following a four minute occlusion of the human forearm.

Kontos et al. (1968a) studied the effect of dipyridamole on the response of the canine hindlimb to a 30 second period of ischemia. This drug potentiates the vasodilator action of adenosine, AMP, and ATP. However, its administration had no effect on the hyperemic response to circulatory arrest.

Tominaga et al. (1973a) used a bioassay preparation to study venous blood following a two minute arterial occlusion in the canine hindlimb. The venous effluent had renal

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vasoconstrictive activity, suggesting that AMP and/or adenosine might be present as these compounds constrict the renal vasculature. Scott et al. (1965) had obtained similar results. Tominaga et al. (1973d) found that venous adenosine and/or AMP rose above control levels following a three minute occlusion in the canine hindlimb. In a comparison of constant pressure and constant flow preparations, Tominaga et al. (1975) found that adenosine and/or AMP increased in the venous blood following a three minute occlusion only with constant flow. They theorized that with constant pressure sufficient blood was supplied to the muscle to effectively wash out mediators and accumulated metabolites, some of which were suppressing the activity of deaminases. Thus, with increased enzyme activity, the adenine compounds would be converted to nonvasoactive products.

The myogenic theory has frequently been used to account for reactive hyperemia. Folkow (1949a), in denervated canine hindlimbs, found vasodilation in response to decreased intravascular pressure. Chemicals did not appear to be the cause, as the blood was not vasoactive when injected into other vessels. Patterson (1956) brought extra blood into the arm by suction and trapped it there during arterial occlusion. This maintained the pressure in the brachial artery distal to the occluding cuff at 45 mm Hg,

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compared with about 10 mm Hg in the non-packed arm. The subsequent hyperemia was considerably less, even after allowing for the extra trapped blood. However, the hyperemia was not completely abolished by this procedure, and the peak flow was changed very little. Freeburg and Hyman (1960) confirmed these results. Therefore, other factors in addition to the myogenic mechanism were considered likely to mediate reactive hyperemia.

Wood et al. (1955) performed a similar series of experiments and obtained similar results. Packing the arm with blood reduced the resultant hyperemia by 20 to 50%, but it was not abolished. Mild exercise during the occlusion increased the reactive hyperemia considerably and was unaffected by maintenance of intravascular pressure.

Kontos et al. (1965) further studied this problem. Venous congestion induced by venous occlusion abolished the vasodilator response to short arterial occlusion (five seconds) but only decreased it in response to a longer arterial occlusion (30 seconds). The reactive hyperemia volume in response to a short occlusion correlated moderately well with the decrease in intravascular pressure. This correlation was poor for a longer occlusion. Owen et al. (1975) found that the recovery time from ischemia progressed with the duration of the ischemia, with both constant pressure and constant flow.

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Therefore, in considering the data, both metabolic and myogenic factors are probably involved in reactive hyperemia. The reactive hyperemia following a short arterial occlusion may be adequately explained by the myogenic hypothesis. However, if the occlusion lasts much longer than five seconds, the opinion is that metabolic factors also play a role in causing the hyperemic response. The most likely metabolic mediators appear to be oxygen, carbon dioxide, and adenine compounds.

1. Surgical Tech

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METHODS

1. Surgical Techniques

Mongrel dogs of both sexes weighing between seven and fifteen kilograms were obtained from the Center for Laboratory Animal Resources and anesthetized initially with 25 mg sodium pentobarbital per kilogram body weight intravenously. A sustaining dosage of six mg per kilogram body weight was administered intraperitoneally when a strong corneal reflex was observed. One gracilis muscle was exposed and surgically isolated. The gracilis nerve was isolated and sectioned. The femoral artery was isolated and all branches between the upper and lower gracilis artery were tied off.* The femoral artery was then tied above the upper gracilis artery and cannulated just distal to the tie with PE 260 tubing, and a constant flow Ringer's perfusate bubbled with a mixture of 95% oxygen, 5% carbon dioxide was immediately commenced via a model T8 Sigmamotor pump. A second occlusive tie was placed on the femoral artery just below the lower gracilis artery. Thus, all perfusate either entered the upper or

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lower gracilis artery. The gracilis vein was cannulated with PE 90 tubing. The origin and insertion of the muscle were tied off. The gracilis nerve was stimulated at either 6V, 6 cps, 1.6 msec or 22V, 10 cps, 1.6 msec with a Grass model S5 stimulator for one minute to wash out residual cells from the muscle vasculature. The muscle was then perfused for an additional 20 minutes before starting an experimental procedure.

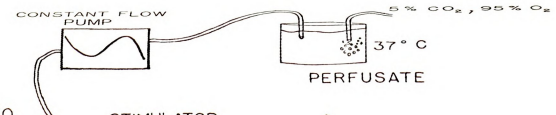
Arterial blood pressure was measured from a femoral artery cannula with a Statham model P23AC pressure transducer and a Grass model 5D polygraph. Flow was adjusted before the start of the experiment to obtain a perfusion pressure as close as possible to 90 mm Hg. Flows ranged from 8 to 11 ml/min. The preparation described above is shown in Figure 1.

2. Ion Exchange Chromatography

A gradient elution ion exchange chromatographic apparatus similar to that described previously by Busch et al. (1952) and Hurlbert et al. (1954) was used to separate the adenine compounds. Three modifications of these procedures were used in the studies described in this thesis. In the first modification a 500 ml reservoir bottle approximately 15 feet from the floor was filled with a 1 M ammonium formate solution. Tubing which could be clamped connected the reservoir bottle to a mixing chamber containing 500 ml

Figure 1. Gracilis muscle preparation. The gracilis muscle preparation shown in this figure and described in Section 1 of Methods was used in all experiments described in this thesis.

*In some preparations the femoral artery was cannulated rather than the gracilis artery. In these latter experiments all branches leading from the femoral except the upper and lower gracilis arteries were tied off.



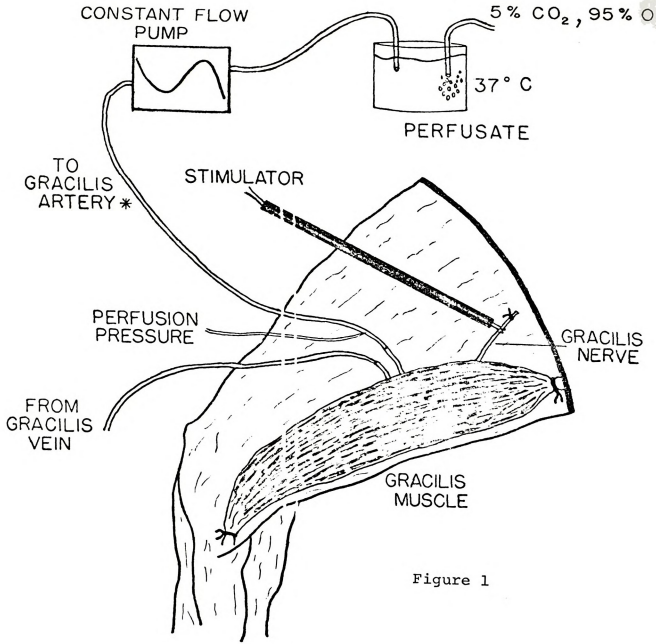

CONSTANT FLOW PUMP
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Figure 1

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of distilled water and a magnetic stirrer. The mixing chamber was approximately 5 feet from the floor and was connected by tubing to a chromatography column of 0.9 cm inner diameter packed with 6.2 cm of Dowex 1 anion exchange resin in the formate form with the positively charged cross-link $\phi CH_2N^+(CH_3)_3$. This tubing could also be clamped when perfusion of the resin column had to be interrupted. As the ammonium formate from the reservoir bottle was gradually added to the mixing chamber, an increasing concentration of ammonium formate was gradually delivered to the resin column. As the slowly increasing ammonium formate solution perfused through the resin column, the adenine nucleotides which had been previously added to the column were sequentially eluted. The resin column was positioned over an automatic fraction collector which contained 90 tubes. The solution flowed from the column into a volumetric siphon which emptied approximately 10 ml aliquots into each test tube of the fraction collector.

Adenosine and other nucleosides, which bind very weakly (if at all) to the resin, came off first, followed in order by AMP, ADP and finally ATP. The reservoir, initially filled with 1 M ammonium formate, was refilled with 500 ml of 1.75 M ammonium formate after fraction collector sample #50 in order to elute the ATP. The apparatus used for this chromatographis separation is shown in Figure 2 and the

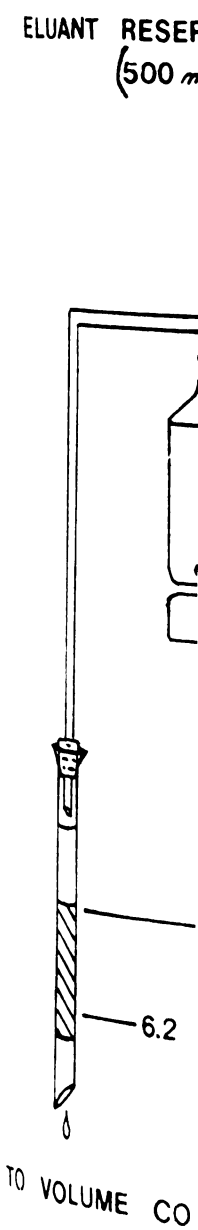
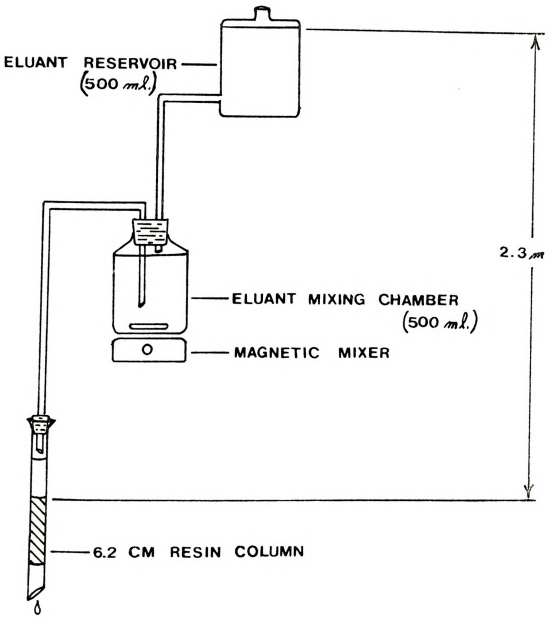


Figure 2. Gradient elution ion exchange column chromatography apparatus. A modification of this apparatus was also used in which Dowex 1 resin in the formate form was packed in a 2 cm inner diameter column to a height of 12.1 cm.


ELUANT RESERVOIR
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2.3 m
ELUANT MIXING CHAMBER
(500 ml.)
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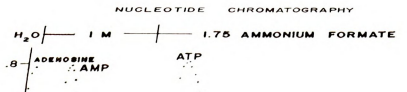
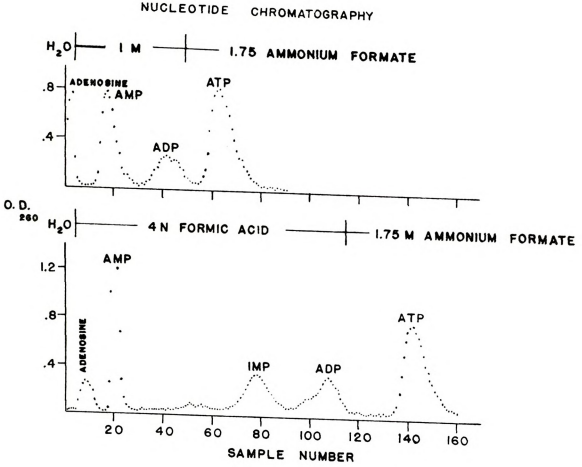
Figure 2

Figure 3. Gradient elution ion exchange chromatography pattern for ATP, ADP, AMP, and adenosine. One-half ml of a solution containing the compounds was added to the column in 2 ml of saline. The apparatus used is shown in Figure 2. Each point represents one observation and was obtained by an optical density reading of the samples at 260 mμ. The lower figure shows the type of separation used to distinguish AMP from IMP when 0.5 ml of a carrier solution containing both AMP and IMP was added to the resin column.

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4 N FORMIC ACID
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Figure 3

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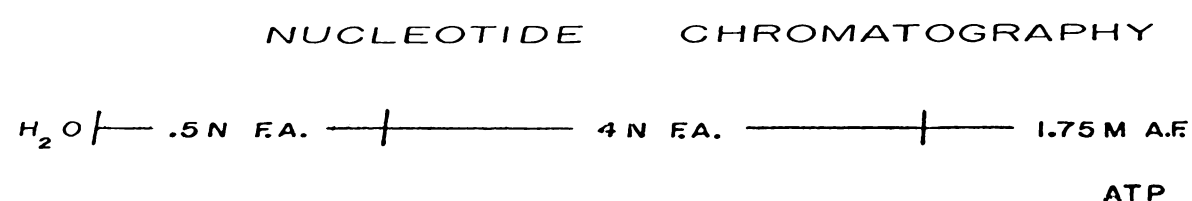
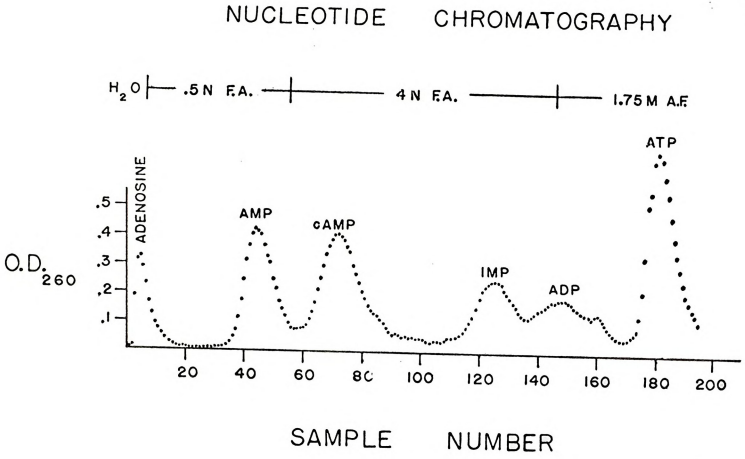
pattern of nucleotide elution is shown in the upper diagram of Figure 3.

Since it was observed that carrier IMP and AMP came off the resin in the same fraction collector samples with the above procedure, a gradient elution procedure utilizing formic acid was modified from that of Hurlbert et al. (1954) to separate AMP from IMP. In this second procedure Dowex I anion exchange resin in the formate form was packed in a 2 cm inner diameter column to a height of 12.1 cm. The elution solution reservoir was filled with 500 ml of 4 N formic acid for the first 120 tubes. At tube 120 the elution solution was changed to 1.75 M ammonium formate to elute ATP. The results of a separation of carrier nucleotides using this system are shown in the lower diagram of Figure 3.

The third modification of this chromatographic procedure was designed to separate cyclic AMP, as well as nucleosides, AMP, IMP, ADP, and ATP from each other. Dowex 1 anion exchange resin was packed in a 2 cm inner diameter column to a height of 12.1 cm. The elution solution reservoir was filled with 500 ml of 0.5 N formic acid for the first fifty tubes. At tube 50 the elution solution was changed to 4 N formic acid. The final change of elution solution was made at tube 145; the reservoir was filled with 500 ml of 1.75 M ammonium formate to elute ATP. The results of a separation of carrier nucleotides using this system are shown in Figure 4.

Figure 4. Gradient elution ion exchange chromatography pattern for ATP, ADP, IMP, cAMP, AMP, and adenosine. One-half ml of a solution containing the compounds was added to the column in 2 ml of saline. Each point represents one observation and was obtained by an optical density reading of the samples at 260 mμ. F.A. = formic acid; A.F. = ammonium formate.




NUCLEOTIDE CHROMATOGRAPHY
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.5N F.A.
4N F.A.
1.75M A.F.
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cAMP
IMP
ADP
ATP
O.D.260
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3. AMP Assay

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For the first separation described, a flow rate of ten ml per minute gave an adequate separation. For the last two separations described, a slower flow rate was necessary to separate the compounds. A flow rate of approximately ten ml per one and one-half minutes was used.

In each of the three procedures described, all samples added to the resin columns were forced onto the resin with approximately 30 ml of distilled water. Optical density readings at 260 mμ on a Beckman DB spectrophotometer were done on all fraction collector samples. In those procedures involving IMP separation, peak readings in the IMP region were re-read at 248 mμ, the wavelength of maximum molar absorbancy of IMP. Recovery of all compounds added to the columns was virtually complete.

3. AMP Assay

An assay was set up to analyze for nanogram levels of AMP which might be present in the venous outflow from tissues. The assay was based on the following reaction:

$$AMP + {}^{14}C\text{-}ATP \xrightleftharpoons{\text{Myokinase}} {}^{14}C\text{-}ADP$$

Myokinase catalyzes the reversible reaction in which one molecule each of AMP and ATP are converted to two molecules of ADP.

To carry out this assay the sample to be tested for AMP was collected and contrifuged at 10,000 x G for five

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minutes at 0° C to remove any cells and platelets which might be present. The supernatant was decanted and three ml of it were pipetted into a test tube. To this sample were added 0.5 ml of TEA buffer containing 0.02 ml of stock myokinase and 1 μCi (1.4 μg) of ^{14}C-ATP in 50 λ of solution. The resulting mixture was gently swirled and then incubated in a shaker water bath at 37° C for 30 minutes. At the end of the incubation period the sample was added to a resin column previously set up for elution of any AMP and ADP which might be present from ^{14}C-ATP, which stayed on the column.

The chromatographic procedure for this assay consists of a column of 0.9 cm inner diameter packed with 1.5 cm of Dowex 1 anion exchange resin in the formate form. Following incubation, a sample was immediately added to the resin and an elution solution of 0.4 M ammonium formate was immediately commenced. The reservoir for the elution solution was positioned approximately three feet above the resin, and the solution flowed onto the resin column via tubing by gravity.

The elution was continued until 150 ml of solution had been collected from the resin column. It had previously been determined that this amount of solution would elute AMP and ADP but not ATP. The sample collected was thoroughly mixed; then one ml was plated on a 1 cm ringed

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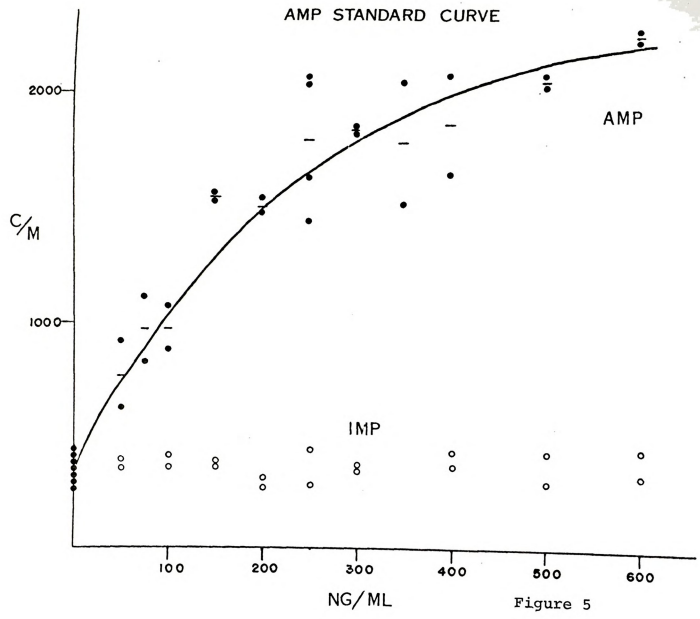
aluminum planchet and dried to infinite thinness. The planchet was then counted in a Nuclear Chicago counter until at least 5,000 counts were registered in order to determine ^{14}C activity.

To determine if the assay was working properly, a standard curve was made each time a change was made in resin, buffer, myokinase, or ^{14}C-ATP. The standard curve was constructed by adding known quantities of AMP to perfusate and then running the samples through the assay described above. Blank samples containing only perfusate, ^{14}C-ATP, buffer and myokinase were also run through the assay. One such standard curve is shown in Figure 5. It can be seen that as sample AMP increases, counts per minute also increase. Therefore, to determine the amount of AMP in an experimental sample, one compares counts per minute obtained by the assay with the corresponding level of AMP on the x-axis. If no AMP was present, counts per minute obtained should have been within the blank range.

Since it was possible that IMP might be released into the venous outflow and since IMP might then react in the AMP assay, the AMP assay was run with known amounts of IMP, as shown in the lower portion of Figure 5. In concentrations up to 600 ng per ml IMP produced no increases in counts per minute above the blank range. It was therefore concluded that the assay was relatively specific for AMP.

Figure 5. AMP standard curve. Samples containing AMP or IMP in known concentrations were run through the AMP assay to determine counts per minute. Closed circles represent AMP samples; open circles represent IMP samples. Each circle represents one observation. Dashes represent the mean counts per minute at a particular concentration of AMP.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NG/ML

Figure 5

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Many of the experimental samples were analyzed for the presence of ATP by the firefly-luciferase method to be described in Section 5. The presence of large quantities of non-labelled ATP in a sample would decrease the specific activity of the ^{14}C-ATP and thereby interfere with the AMP assay, as a decrease in counts per minute obtained would result.

4. ^{14}C-ATP Purity Check

A purity check was done on each batch of ^{14}C-ATP used by adding 1 μCi (1.4 μg) of the isotope in 2 ml of saline with 0.5 ml of carrier nucleotides to the resin column described in the first part of Section 2. The sample was chromatographically separated into ATP, ADP, AMP, and nucleosides. One ml of each fraction collector sample was plated on a 1 cm aluminum planchet and dried to infinite thinness. Each planchet was then counted in a Nuclear Chicago counter for one minute to determine ^{14}C-activity. Identification of ^{14}C-labeled adenine compounds present was inferred by comparison of ^{14}C-labeled peaks with carrier nucleotide peaks determined by optical density readings. Greater than ninety per cent of the total ^{14}C activity in the sample was ^{14}C-ATP. ADP, AMP, and nucleosides each accounted for approximately 2 per cent of the total ^{14}C activity added to the resin column.

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5. Firefly-Luciferase Assay for ATP

A technique was set up to detect nanogram levels of ATP. The method was a modification of the bioluminescence technique described by Silinsky (1974). The assay depends upon the emission of light which occurs when ATP is added to firefly lantern extract (luciferase). The amount of light emitted is directly proportional to the ATP concentration. This method is very sensitive, having the ability to detect as little as one nanogram per ml of ATP.

The light detection system consisted of a Fluke 415B high voltage power supply connected to the input of a photomultiplier tube. The tube output was amplified and permanently recorded by a Honeywell apparatus. The photomultiplier tube and reaction chamber were enclosed to prevent the entrance of light during operation. The reaction chamber consisted of a holder for the four ml reaction curvette and a stable holder for the syringe containing the sample.

The procedure for testing a sample for ATP was as follows. One-half ml of luciferase solution was placed in a reaction cuvette. The cuvette was then placed inside the reaction chamber. All lights in the room were turned out during this procedure except a dim red light for illuminating the recorder. A one ml syringe filled with exactly 0.25 ml of sample was positioned in its holder above the

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cuvette. The high voltage power source and the recorder were activated. The sample was then injected into the cuvette as rapidly as possible. Any ATP present in the sample was detected as light emission and recorded as a deflection of the pen on the Honeywell recorder.

To quantify the amount of ATP which a sample might contain, an ATP standard of approximately 10 ng per ml was run before and after each experimental series. The maximum deflection produced by each sample was compared with the maximum deflection produced by the standards. Standard curves had been made which demonstrated the linear relation between ATP concentration in perfusate and amount of pen deflection. The correlation coefficient between ATP concentration and cm deflection was 0.86 for 47 samples.

This technique was used to test ATP content in approximately one-third of the samples run through the AMP assay. The method was also used during the intraarterial infusion of various concentrations of ATP into the gracilis muscle to determine effluent ATP concentration.

6. Infusion of Nucleotides into the Gracilis Muscle to Determine Uptake and Breakdown Products

Nucleotides were infused intraarterially into the gracilis muscle and the effluent collected for chromatographic identification of breakdown products arising from

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the specific nucleotide infused. Carrier ATP was infused at 100 µg per ml. Twenty ml of the effluent were collected and analyzed by the chromatographic separation shown in Figure 4. In some experiments ^{14}C-ATP was infused at 500 ng per ml (7 µCi); ten ml of both inflow and outflow were collected and analyzed by the chromatographic separation shown in the upper part of Figure 3. ADP was infused at 70 µg per ml, and 40 ml of both inflow and outflow were collected and analyzed by the chromatographic separation shown in Figure 4. IMP was infused at 100 µg per ml, and 25 ml of the effluent were collected and analyzed by the chromatographic separation shown in the upper part of Figure 3. AMP was infused at 100 µg per ml; ten ml of the effluent were collected and analyzed by the chromatographic separation shown in the lower part of Figure 3.

A series of three experiments was done to determine the effect of flow rate on AMP survival in passing through the gracilis muscle vasculature. AMP was infused intraarterially at 100 µg per ml. Ten ml of effluent were collected both at a slow flow rate of approximately three ml per minute and at a fast flow rate of approximately ten ml per minute. Samples were analyzed by the chromatographic procedure shown in the lower part of Figure 3.

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and comparing their absorbance at 260 mμ on a spectrophotometer. Uptake of ^{14}C was determined by making a 1:25 dilution of both inflow and outflow. One ml of each dilution was plated, dried, and counted for ten minutes. A comparison of total counts gave uptake; total outflow counts divided by total inflow counts times 100 gave per cent not taken up by the muscle.

7. Studies of the Effect of Arterial Occlusion on Venous AMP

Two controls* were collected, followed by a two minute occlusion of inflow. Immediately upon starting the flow two more samples were collected. Fifteen minutes later a control was collected, followed by a three minute stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec. Two samples were collected during the stimulation. A final control was collected ten minutes after the end of the stimulation. All samples were analyzed by the AMP assay.

8. Studies of the Effect of Nerve Stimulation on Venous AMP

For the series at 6V, 6 cps, 1.6 msec a control was collected. The gracilis nerve was then stimulated for two and one-half minutes, during which time one sample was

*Controls were effluent samples collected at times when no experimental treatment of any sort was applied to the muscle.

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collected. Another control was collected five minutes after the end of the stimulation. For the series at 5V, 2 cps, 0.5 msec two controls were collected, followed by a 20 minute stimulation of the gracilis nerve. Muscle tension was measured in this series with a strain gauge transducer and recorded on a Grass polygraph. Four samples were collected during the stimulation at periodic intervals. Final controls were collected first immediately after the stimulation and another ten minutes after the stimulation. All samples collected were analyzed by the AMP assay.

9. Studies of the Effect of Isoproterenol on Venous AMP

Following collection of two controls, isoproterenol was infused intraarterially at 500 ng per minute into the gracilis muscle. At the first indication of vasodilation an effluent sample was collected, followed immediately by a second collection. Fifteen minutes after the end of the infusion a control was collected, followed by a five minute stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec. Two samples were collected during the stimulation. One final control was collected five minutes after the end of the stimulation. Each effluent sample collected was analyzed by the AMP assay.

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10. Studies on the Effect of Nerve Stimulation on Venous AMP during Gallamine Infusion

Two series of experiments were performed with gallamine. In one the stimulation parameters were 6V, 6 cps, 1.6 msec. In the other the parameters were 5V, 2 cps, 0.5 msec. The experimental protocol was identical for the two series. Two controls were collected, followed by infusion of 240 µg per minute of gallamine. A third control was collected just after the start of the infusion. Twenty minutes later a fourth control was collected. Then the gracilis nerve was stimulated for five minutes, during which time two samples were collected. Ten minutes after the end of the infusion a control was collected, followed by a two-minute nerve stimulation. One sample was collected during this final stimulation. Each effluent sample collected was analyzed by the AMP assay. Muscle tension in both series was measured with a strain gauge transducer.

11. Studies on the Effect of Nerve Stimulation on Venous AMP during Curare Infusion

Two series were performed with curare. In the first series two controls were collected, followed by the intra-arterial infusion of curare at 30 µg per minute into the gracilis muscle. A third control was collected just after the start of the infusion. Twenty-five minutes later a

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fourth control was collected. The gracilis nerve was then stimulated at 5V, 2 cps, 1.6 msec for five minutes, during which time two samples were collected. Two final controls were collected approximately 20 minutes after the end of the stimulation. In the second series two controls were collected followed by intraarterial infusion of curare at 30 µg per minute into the gracilis muscle. A third control was collected just after the start of the infusion. Twenty minutes later a fourth control was collected, followed by gracilis nerve stimulation at 20V, 10 cps, 1.6 msec for five minutes. Two samples were collected during the stimulation. Ten minutes after the end of the stimulation a final control was collected. Each effluent sample collected was analyzed by the AMP assay. Muscle tension was measured with a strain gauge transducer in both curare series.

12. Studies on the Effect of Nerve Stimulation on Venous AMP during Phentolamine Infusion

Following collection of two controls, phentolamine was infused into the gracilis muscle intraarterially at 50 µg per minute. A third control was collected just after the start of the infusion. Ten minutes later a fourth control was collected, followed by a three-minute stimulation of the gracilis nerve at 22V, 10 cps, 1.6 msec. Two samples were collected during the stimulation. Each effluent sample collected was analyzed by the AMP assay. To demonstrate

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13. Studies on the Effect of Norepinephrine and ADH Infusion on Venous AMP

The vasoconstrictor agents norepinephrine and ADH were intraarterially infused into the gracilis muscle to determine their effect on venous AMP. Norepinephrine was infused at 760 ng per minute, and ADH was infused at 0.06 units per minute. The experimental protocol was identical for the two series. Two controls were collected, followed immediately by infusion of the drug. Three samples were collected at periodic intervals during the infusion. One final control was collected ten minutes after the infusion was stopped. Each venous sample collected was analyzed by the AMP assay. To demonstrate that the presence of norepinephrine or ADH alone in the AMP assay did not produce counts per minute above the blank range, inflow samples containing only perfusate and norepinephrine or ADH were run through the AMP assay. Counts per minute obtained with both drugs were within the blank range.

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14. Studies on the Effect of Norepinephrine on Venous AMP during Phentolamine Infusion

Following collection of two controls phentolamine was infused into the gracilis muscle at 50 µg per minute for ten minutes. Two additional controls were collected at the beginning and the end of this period. Then norepinephrine, 760 ng per minute, was infused concurrently with the phentolamine for twelve minutes. Two samples were collected. Each effluent sample collected was analyzed by the AMP assay.

15. Studies on the Effect of Epinephrine Infusion on Venous AMP

Two series were done in which epinephrine was infused intraarterially into the gracilis muscle. In the first series two controls were collected, followed by the infusion of epinephrine at 1.5 µg per minute for ten minutes. Two samples were collected during this time. Phentolamine was then infused at 50 µg per minute concurrently with the epinephrine for ten minutes. Two additional samples were collected. In the second series two controls were collected, followed by the infusion of epinephrine at 61 ng per minute for fifteen minutes. Three samples were collected at intervals during this infusion. One final control was collected ten minutes after the infusion was stopped. Each effluent sample collected was analyzed by the AMP assay.

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17. Reagents

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To demonstrate that epinephrine alone would not increase counts per minute above the blank range, inflow samples containing only perfusate and epinephrine at the higher dose were run through the AMP assay. Counts per minute obtained were within the blank range.

16. Studies on the Effect of Ouabain Infusion on Venous AMP

Following collection of two controls ouabain was infused intraarterially into the gracilis muscle at doses ranging from 75 µg per minute to 225 µg per minute for 20 minutes. Four samples were collected at periodic intervals during this infusion. One final control was collected ten minutes after the infusion was stopped. Each effluent sample collected was analyzed by the AMP assay. No experiments were done to determine if ouabain inhibited the AMP assay.

17. Reagents

1. Ringer's Perfusate.--One liter of distilled water was added to the following compounds:

KH_2PO_4	.07 gm
$CaCl_2 \cdot 2H_2O$	.4 gm
KCl	.3 gm
$MgCl_2 \cdot 6H_2O$	.2 gm
glucose	.9 gm
pyruvate	.22 gm
NaCl	7.0 gm

Finally, 1.8
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Finally, 1.8 gm of $NaHCO_3$ were added to the above solution. After rapid mixing the solution was immediately placed in a 37.5°C constant temperature water bath and bubbled with a mixture of 95 per cent oxygen, 5 per cent carbon dioxide.

2. ^{14}C-ATP.--Uniformly labeled ^{14}C-ATP was obtained from New England Nuclear (Lot #893-209; 0.31 mg ATP/12.5 ml 50 per cent ethanol; specific activity 417 mCi/mM; and Lot #893-098; 0.37 mg ATP/12.5 ml 50 per cent ethanol; specific activity 385 mCi/mM). The solution was shipped in a dry ice container and stored as suggested by New England Nuclear at -15°C. Purity checks were made on each lot of ^{14}C-ATP; however, the specific activities were not checked in our laboratory.

3. Carrier Nucleotides.--Adenosine, Ba-ADP, cyclic AMP, and IMP were obtained from Sigma Chemical Company. ATP and AMP were obtained from Nutritional Biochemical Corporation. All were stored at -15°C. A solution of carrier nucleotides for determination of ion exchange chromatographic peak location was prepared in the following way. Five mg adenosine, 20 mg AMP, 30 mg cyclic AMP, 35 mg IMP, and 60 mg ATP were mixed with 4 ml distilled water. The solution was heated slightly with hot tap water to dissolve the AMP completely. To convert insoluble Ba-ADP to soluble Na-ADP, twenty-five mg of Ba-ADP were mixed with 4 ml of 0.2 M sodium sulfate. The solution was then centrifuged for two minutes.

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The supernatant was decanted and added to the nucleotide solution described above. The resulting solution thus contained 0.6 mg/ml adenosine, 2.5 mg/ml AMP, 3.7 mg/ml cyclic AMP, 4.4 mg/ml IMP, 3.1 mg/ml ADP, and 7.5 mg/ml ATP. One-half ml of this solution was added to a column for fractionation by ion exchange chromatography.

4. Ammonium Formate.--A 1.75 M ammonium formate elution solution was prepared by adding 18 liters of distilled water to 1980 gm of reagent grade ammonium formate obtained from Matheson, Coleman and Bell Company. Reagent grade formic acid obtained from Mallinckrodt Company was added to this solution until pH 5 was reached. The pH was measured on a Beckman Expandomatic pH meter equipped with external electrodes.

One molar ammonium formate elution solution was prepared by adding 857 ml of distilled water to 1143 ml of 1.75 M ammonium formate.

5. 4 N Formic Acid.--A 4 N formic acid elution solution for gradient elution ion exchange chromatography was prepared by diluting 209 gm of 88 per cent formic acid to 1 liter with distilled water.

6. 0.5 N Formic Acid.--A 0.5 N formic acid elution solution was prepared by adding 125 ml of 4 N formic acid to 875 ml of distilled water.

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7. Luciferase.--Luciferase was obtained from Sigma Chemical Company and stored at -15°C. Five ml of perfusate were added to each bottle. One-half ml of the resulting solution was used per ATP analysis. The mixture was swirled each time before use.

8. ATP Standard.--An ATP standard for the luciferase assay was prepared by adding 20 mg of ATP to 100 ml of distilled water. One-tenth ml of this solution was then added to 100 ml of distilled water. Finally, 0.5 ml of this second solution were added to 9.5 ml of perfusate. ATP concentration of this standard was approximately 10 ng/ml.

9. 0.1 M TEA Buffer.--TEA buffer was prepared by adding 18.6 gm of triethanolamine obtained from Sigma Chemical Company and 0.2 gm of $MgCl_2 \cdot 6H_2O$ to 1 liter of distilled water. The pH was then adjusted to 7.8 with 1 N NaOH. 0.2 ml of myokinase was added to five ml of this buffer, and one-half ml of the resulting solution was used for each AMP assay.

10. Myokinase.--Stock rabbit muscle myokinase (Lot 320038) was obtained from Calbiochem and refrigerated. For each AMP assay performed, 0.02 ml of stock enzyme was added. The stock enzyme contained 1890 I.U./ml.

11. Isoproterenol.--Isoproterenol was obtained from Sigma Chemical Company. An infusion solution was prepared

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by adding 0.5 mg isoproterenol to 50 ml saline. The resulting solution was infused at 0.05 ml per minute intraarterially.

12. Curare.--Curare was obtained from Eli Lily Company and refrigerated. Three mg of curare were added to 50 ml of perfusate. The resulting solution was infused at 0.5 ml per minute intraarterially.

13. Gallamine.--Gallamine triethiodide was obtained from Davis and Geck Company. Ten mg of gallamine were added to 50 ml of perfusate. The resulting solution was infused at 1.2 ml per minute intraarterially.

14. Phentolamine.--Phentolamine mesylate was obtained from CIBA Pharmaceutical Company. Five mg of phentolamine were added to 20 ml of perfusate. The resulting solution was infused at 0.2 ml per minute intraarterially.

15. Norepinephrine.--Norepinephrine was obtained from Sigma Chemical Company. Thirty mg of norepinephrine were added to 100 ml of distilled water; 0.2 ml of this solution was then added to 60 ml of perfusate. The resulting 1 µg/ml solution was infused at 0.76 ml per minute intraarterially.

16. ADH.--ADH was obtained from Sigma Chemical Company. Four units of ADH were added to 50 ml of perfusate. The resulting solution was infused at 0.76 ml per minute intraarterially.

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17. Epinephrine.--Adrenalin chloride solution was obtained from Parke, Davis and Company. A low dose infusion was prepared by adding one mg of epinephrine to 100 ml of distilled water. Eight ml of this solution were then added to 32 ml of distilled water. Two ml of this solution were added to 50 ml of perfusate. The resulting 80 ng per ml solution was infused at 0.76 ml per minute. A high dose infusion was prepared by adding one mg of epinephrine to 100 ml of distilled water. Eight ml of this solution were then added to 32 ml of perfusate. The resulting 2 µg per ml solution was infused at 0.76 ml per minute intraarterially.

18. Ouabain.--Ouabain was obtained from Nutritional Biochemical Corporation. Five mg of ouabain were added to 50 ml of perfusate. Following a one minute sonification, the solution was infused at 0.76 ml per minute intraarterially.

19. ATP Infusion.--ATP obtained from Nutritional Biochemical Corporation was added to perfusate in the amount of 10 mg per 100 ml.

20. ADP Infusion.--Sixty mg of Ba-ADP obtained from Sigma Chemical Company were added to 8 ml of 0.2 M sodium sulfate. The resulting solution was stirred and then centrifuged for two minutes. The supernatant was decanted and added to approximately 800 ml of perfusate.

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21. AMP Infusion.--AMP obtained from Nutritional Biochemical Corporation was added to perfusate in the amount of 10 mg per 100 ml.

22. ^{14}C-ATP Infusion.--One-half ml of ^{14}C-ATP (14 µg; 10 µCi) was added to 30 ml of perfusate.

23. Resin Regeneration.--The resin used for gradient elution ion exchange chromatography was washed three times in 88% formic acid by soaking overnight and decanting the supernatant each time. The resin was then washed approximately 20 times with distilled water. The resin used for the AMP assay was washed three times in a 1:1 mixture of 30% ethanol and 1 N HCl by soaking overnight and decanting the supernatant each time. This resin was then further washed three times in 88% formic acid as described above and was finally washed approximately 20 times with distilled water.

18. Statistical Methods

Data from nerve stimulation plus phentolamine and low voltage nerve stimulation experiments was analyzed by a completely randomized one-way analysis of variance for unequal sample size. Data from low dose epinephrine, norepinephrine plus phentolamine, and curare plus low voltage nerve stimulation experiments was analyzed by a randomized complete block model analysis of variance. Prior to the analysis of variance the sample mean variances were

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determined to be homogenous by using the F max test. Data from the remaining experiments in which AMP assays were done was analyzed by the Student's t-test modified for paired replicates. Initial, non-experimental values served as statistical controls. A linear regression analysis was performed on all venous AMP concentrations and their corresponding perfusion pressures. The correlation coefficient was determined.

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RESULTS

1. ATP Survival on Passage through the Gracilis Muscle Vasculature

To determine whether or not ATP can survive passage through the gracilis muscle vasculature, six experiments were performed in which increasing concentrations of ATP were added to the Ringer's perfusate and infused into the gracilis artery. The venous effluent was analyzed by the firefly-luciferase method for ATP concentration. Figure 6 shows the results of these experiments. It can be seen that virtually no ATP appears in the venous effluent when dilatory levels (0.5 µg/ml) of ATP are infused intraarterially. With infusion concentrations of ATP of 10 µg/ml and higher, ATP can be detected in the venous effluent in increasing amounts. Although flow rate varied from 8 to 11 ml/min. between preparations, it is considered unlikely that this small variability could have produced such consistent results as were observed in this series of experiments. These results indicate that it would be almost impossible to quantify low levels of ATP which might be released from within the gracilis muscle into the perfusate during active or reactive hyperemia. Approximately one-third of the

Figure 6. ATP survival on passage through the gracilis muscle vasculature. ATP was infused intraarterially into the gracilis muscle in varying concentrations. The venous effluent was analyzed by the firefly-luciferase assay for ATP concentration. Each type of symbol represents the data from one animal, and each symbol represents one venous ATP assay. Arterial ATP concentration is plotted on a log scale. N = 6

ATP SURVIVAL ON PASSAGE THROUGH GRACILIS MUSCLE

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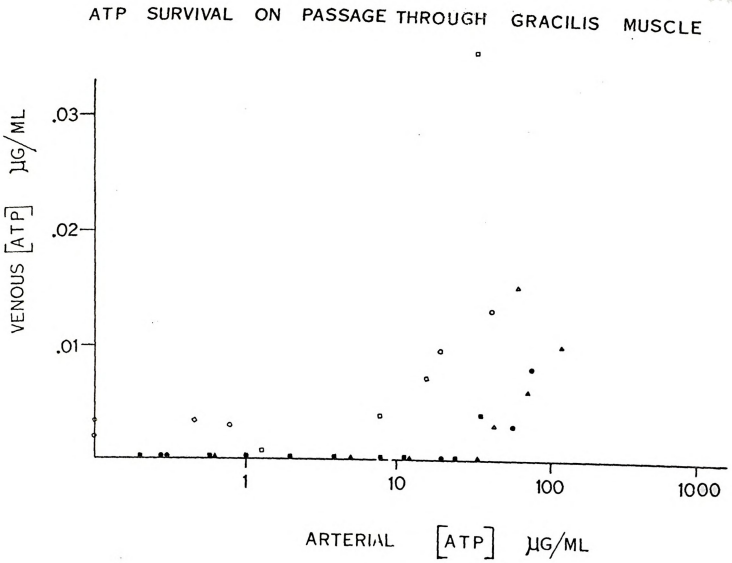
ATP SURVIVAL ON PASSAGE THROUGH GRACILIS MUSCLE
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effluent samples which were analyzed by the AMP assay, as described throughout this Results section, were also tested for ATP by the firefly assay. No significant quantity (greater than 10 ng/ml) of ATP was ever detected in any sample.

2. Breakdown Products of ATP on Passage through the Gracilis Muscle Vasculature

To determine breakdown products of ATP on passage through the gracilis muscle vasculature 100 µg ATP per ml of perfusate were infused intraarterially into the gracilis muscle. Figure 7 shows the results of five such experiments. The venous effluent was collected and analyzed by ion exchange chromatography for ATP breakdown products. Effluent $O.D._{260}$ was determined to be constant before sample collection. It can be seen that AMP is the major breakdown product of ATP in the gracilis muscle vasculature. A small peak is present in the nucleoside area.

Figure 8 shows the results of infusing 500 ng/ml of ^{14}C-ATP through the gracilis muscle. The use of labelled ATP enabled breakdown products to be determindd with infusion of very low levels of ATP. Two experiments were performed. The major breakdown product again is AMP, even though inflow ATP concentration here is very small.

Figure 7. Breakdown products of ATP on passage through the gracilis muscle vasculature. ATP was infused intraarterially into the gracilis muscle at 100 µg/ml. The venous effluent was collected and analyzed by the ion exchange chromatographic procedure shown in Figure 4. Each fraction collector sample was read at 260 mµ on a Beckman BD Spectrophotometer. Each point represents one observation. N = 5

BREAKDOWN PRODUCTS OF ATP

BREAKDOWN PRODUCTS OF ATP

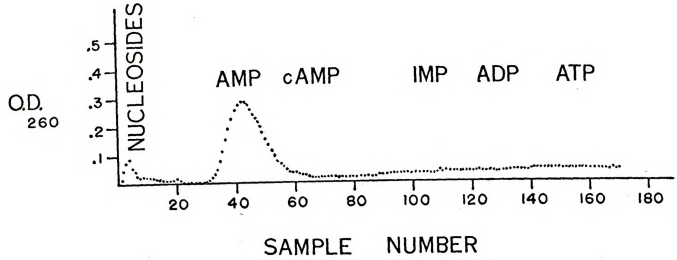

Figure 7

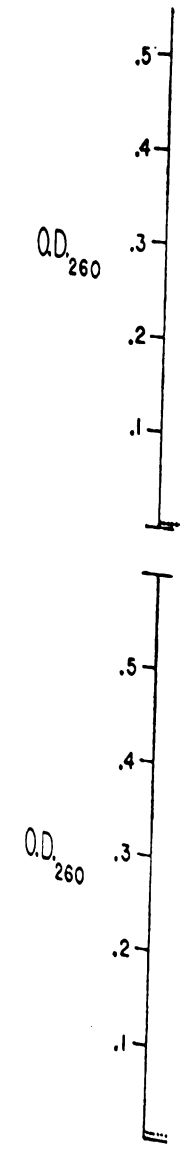

Figure 8. Breakdown products of ^{14}C-ATP on passage through the gracilis muscle vasculature. ^{14}C-ATP (500 ng/ml; 7 μCi) was infused intra-arterially into the gracilis muscle. Eight ml of both inflow and outflow, after the addition of 1/2 ml of carrier nucleotides to each, were added to columns for the ion exchange chromatographic separation described in the upper part of Figure 3. One ml of each fraction collector sample was plated, dried, and counted for one minute. Each point represents one observation.

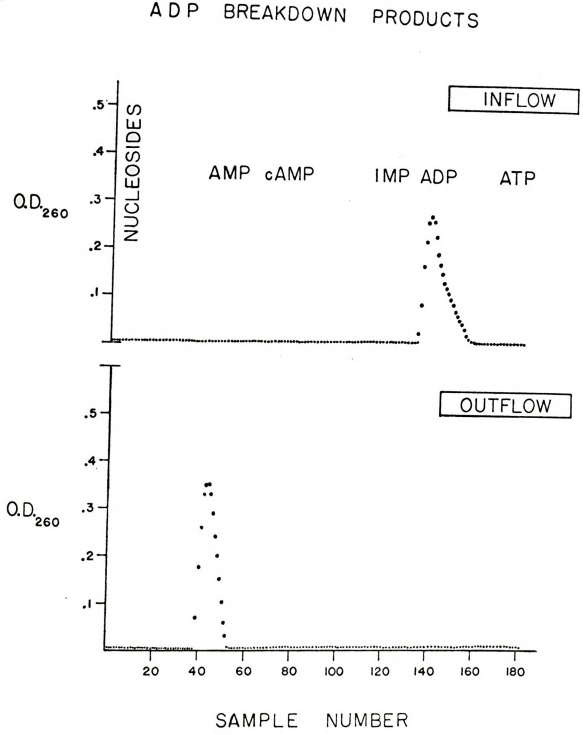
ADP BREAKDOWN PRODUCTS
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Figure 8

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Table 1 shows the specific results of all seven of these experiments. In both series of experiments less than 15% uptake of the ATP degradation products occurred; recovery of the adenine molecule was approximately 90%, as can be seen in Table 2.

3. Breakdown Products of ADP on Passage Through the Gracilis Muscle Vasculature

ADP at a concentration of approximately 70 µg per ml was passed through the gracilis muscle vasculature in three experiments. Venous effluent was collected and analyzed by ion exchange chromatography for ADP breakdown products. Figure 9 shows that virtually all of the ADP infused was degraded to AMP on a single pass through the muscle. Table 3 shows that only 8% uptake of the adenine ring occurred. Therefore, release of either ATP or ADP in the muscle would appear in the effluent as AMP.

4. Survival of AMP on Passage through the Gracilis Muscle Vasculature

In five experiments 100 µg AMP/ml were infused intra-arterially into the gracilis muscle. The venous effluent was collected and analyzed for adenine compounds by the ion exchange chromatographic procedure described in the lower portion of Figure 3. Effluent $O.D._{260}$ was determined to be constant before sample collection. The results are shown in Figure 10.

Table 1. Br

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Table 1. Breakdown products of ATP.

Exp. #	% of Total Recovered From Resin Column			
	Nucleosides	AMP	ADP	ATP
1*	5	86	1	8
2*	4	91	3	2
3	23	77	0	0
4	14	86	0	0
5	14	86	0	0
6	6	94	0	0
7	4	96	0	0
Mean	10	88	0.57	1.43
S.E.	2.73	2.4	0.43	1.13

*^{14}C-ATP infused

Table 2. Change in concentration of the adenine ring of ATP on passage through the gracilis muscle.

Exp. #	Inflow O.D.$_{260}$	Outflow O.D.$_{260}$	% Change
1	.221	.240	+8
2	.213	.192	-10
3	.201	.201	0
4	.283	.257	-9
5	.230	.222	-4
6*	26028 c/m	21737 c/m	-15
7*	22749 c/m	18943 c/m	-18
		Mean =	-6.86
		S.E. =	3.38

*^{14}C-ATP infused

Figure 9. Breakdown products of ADP on passage through the gracilis muscle vasculature. ADP was infused intraarterially into the gracilis muscle at a concentration of 70 µg/ml. The venous effluent was collected and analyzed by the ion exchange chromatographic procedure shown in Figure 4. Each fraction collector sample was read at 260 mµ on a Beckman DB Spectrophotometer. Analysis of the inflowing perfusate in this manner is shown in the upper portion of this figure. Each point represents one observation. N = 3

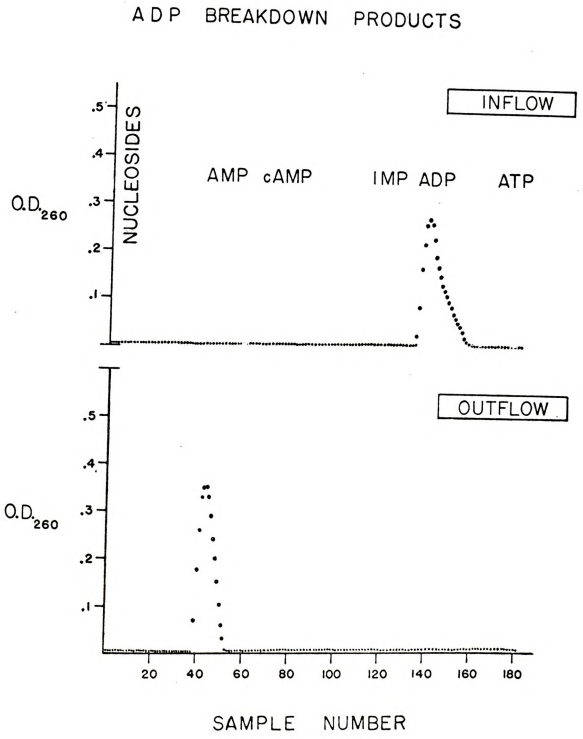
ADP BREAKDOWN PRODUCTS
INFLOW
NUCLEOSIDES
AMP cAMP
IMP ADP
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Figure 9

Table 3. C
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Table 4. C
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Exp. #	
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Table 3. Change in concentration of the adenine ring of ADP on passage through the gracilis muscle.

Exp. #	Inflow $O.D._{260}$	Outflow $O.D._{260}$	% Change
1	.150	.145	-3
2	.105	.095	-9
3	.160	.140	-12
		Mean =	-8
		S.E. =	5.3

Table 4. Change in concentration of the adenine ring of AMP on passage through the gracilis muscle.

Exp. #	Inflow $O.D._{260}$	Outflow $O.D._{260}$	% Change
1	.310	.288	-7
2	.422	.370	-12
3	.380	.340	-11
4	.427	.420	-2
5	.368	.345	-6
		Mean =	-7.6
		S.E. =	1.81

Figure 10. AMP survival on passage through the gracilis muscle vasculature. AMP was infused intraarterially into the gracilis muscle at a concentration of 100 µg/ml. The venous effluent was collected and analyzed by the ion exchange chromatographic procedure shown in the lower portion of Figure 3. Each fraction collector sample was read at 260 mµ on a Beckman DB Spectrophotometer. Each point represents one observation. N = 5

AMP SURVIVAL

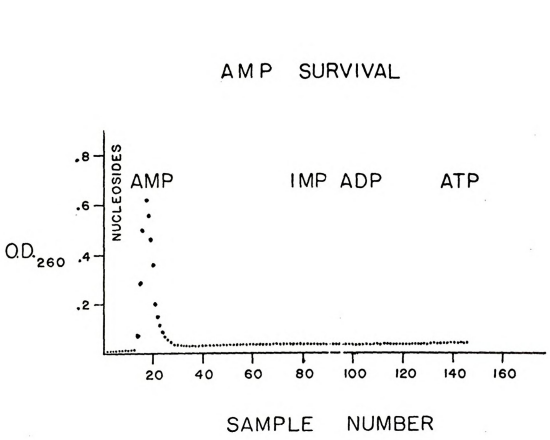
AMP SURVIVAL
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Figure 10

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Table 5.

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virtually all of the AMP infused was able to pass out of the muscle intact. Mean adenine uptake by the muscle was only 7.6%, as shown in Table 4 (page 113). Therefore, ATP, ADP, and AMP released within the muscle would all appear in the venous effluent as AMP.

5. Effect of Flow Rate on AMP during Passage through the Gracilis Muscle Vasculature

Three experiments were performed to determine the effect of flow rate on AMP breakdown and uptake by the gracilis muscle. The results are presented in Table 5.

Table 5. Effect of flow rate on AMP during passage through the gracilis muscle vasculature.

	PER CENT UPTAKE*	
	Slow Flow	Fast Flow
	14	3
	14	9
	12	4
Mean	13.3	5.3
	PER CENT $O.D._{260}$ IN NUCLEOSIDE AREA	
	Slow Flow	Fast Flow
	13	6
	7	9
	12.5	5
Mean	10.8	6.7

*Based on $O.D._{260}$

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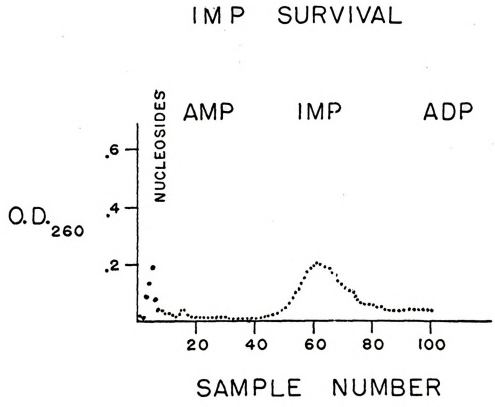
At a flow rate of approximately 10 ml/minute, mean uptake was 5.3 per cent. When flow rate was decreased to approximately 3 ml/minute, mean uptake increased to 13.3 per cent. The effect of flow rate on nucleoside formation was not as consistent. In two of the three experiments nucleoside formation did increase with low flow. In the third there was little difference in nucleoside formation between the two flow rates. At fast flow a mean of 6.7 per cent of the total $O.D._{260}$ was in the nucleoside area, whereas at slow flow a mean of 10.8 per cent was in this area. This indicates that nucleoside formation or accumulation may be greater with slower flow rates, thus accounting for greater adenine ring uptake at low flow.

6. Survival of IMP on Passage through the Gracilis Muscle Vasculature

In one experiment 100 µg/ml of IMP were intraarterially infused into the gracilis muscle. The venous effluent was analyzed by the ion exchange chromatographic procedure shown in the lower portion of Figure 3. Figure 11 shows that IMP is able to pass through the muscle vasculature intact. The small peak in the nucleoside area may be due to the presence of hemoglobin and/or dextran in these samples rather than nucleosides, since these two substances would not bind to the resin and would therefore elute at once. Some cells were noted in these samples and dextran was

Figure 11. IMP survival on passage through the gracilis muscle vasculature. IMP was infused intra-arterially into the gracilis muscle at a concentration of 100 μg/ml. The venous effluent was collected and analyzed by the ion exchange chromatographic procedure shown in the lower portion of Figure 3. Each fraction collector sample was read at 260 mμ on a Beckman DB Spectrophotometer. Each point represents one observation. N = 1

O.D.$_{260}$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present in the perfusate in this experiment. No uptake of IMP was found. Thus, if extracellular IMP had formed in the above studies with adenine nucleotides, it would have appeared in the venous effluent. These studies also indicate that venous AMP cannot come from extracellular IMP.

7. Effect of Occlusion on AMP Outflow

Figure 12 shows the results of a two minute occlusion of the perfusate on effluent AMP. It appears from this figure that AMP efflux did increase immediately following occlusion. However, an increase occurred in only three of the nine experiments of this type; in the remaining six no increase was observed. Statistical analysis of the data showed no significant difference between the second control and the first post-occlusion sample. Perfusion pressure decreased in six of the nine experiments, indicating vasodilation. In only two of the six experiments with vasodilation did effluent AMP levels rise.

In contrast, muscular exercise produced by stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec did produce a statistically significant increase in AMP efflux. However, the increase occurred in only six of the nine experiments and was quite small in most cases. The reason for such a weak response may be that the stimulation parameters 6V, 6 cps, 1.6 msec are borderline, producing weak sympathetic stimulation in some preparations and little or none in

Figure 12. Effect of occlusion and nerve stimulation on venous AMP. Following collection of two control samples, flow to the gracilis muscle was stopped for two minutes. Immediately after flow was resumed, two samples were collected. Fifteen minutes later a control was collected, followed by a three minute stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec. Two samples were collected during this stimulation. A final control was collected ten minutes after the end of the stimulation. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of nine experiments.

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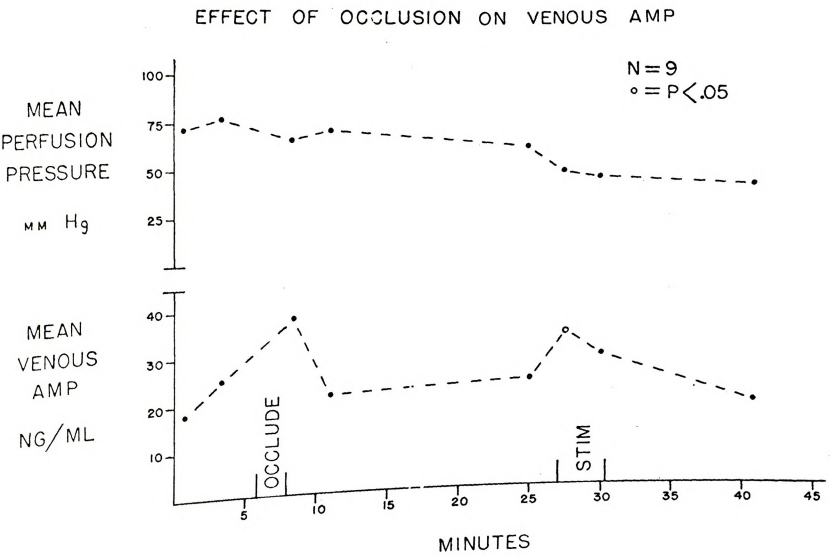

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others. The drop in perfusion pressure during the stimulation period indicates that this may indeed be the case, as sympathetic stimulation would produce vasoconstriction. No significant increase in AMP outflow was found in the second stimulation sample. It is also not likely that blood cells in the effluent gave rise to the AMP observed, since the amount of cells and AMP did not correlate. Also, ATP assays were done on many of the samples and no significant quantities were found.

8. Effect of Nerve Stimulation at 6V, 6 cps, 1.6 msec on AMP Outflow

A series of 11 experiments was done to determine the effect of muscular exercise produced by stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec on AMP outflow. The results, presented in Figure 13, show that AMP outflow did significantly increase above control levels during the stimulation period. The increase occurred in ten of the 11 experiments. The small size of the increase may again be due to borderline stimulation parameters. Effluent AMP levels returned to control values immediately following the stimulation. Perfusion pressure increased during the stimulation in seven experiments, indicating sympathetic nerve activity. In three other experiments pressure remained at control levels during the stimulation, also possibly indicating sympathetic activity.

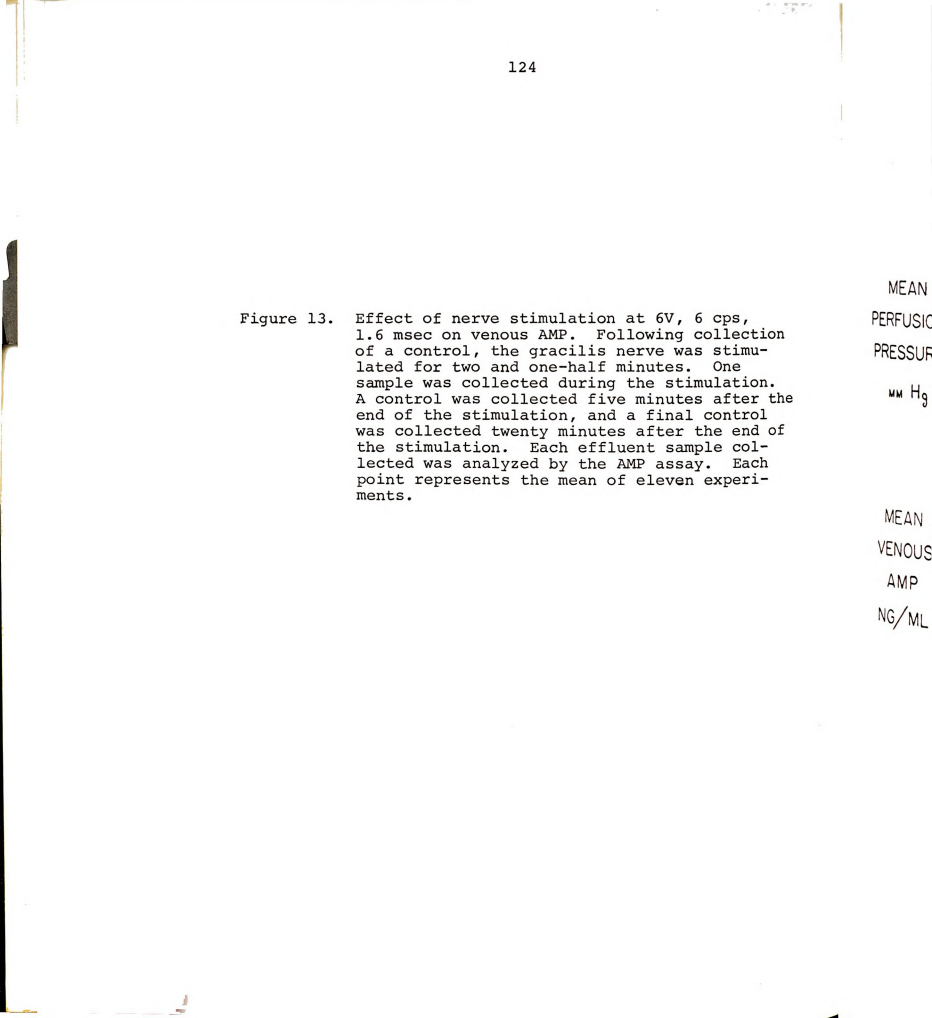

Figure 13. Effect of nerve stimulation at 6V, 6 cps, 1.6 msec on venous AMP. Following collection of a control, the gracilis nerve was stimulated for two and one-half minutes. One sample was collected during the stimulation. A control was collected five minutes after the end of the stimulation, and a final control was collected twenty minutes after the end of the stimulation. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of eleven experiments.

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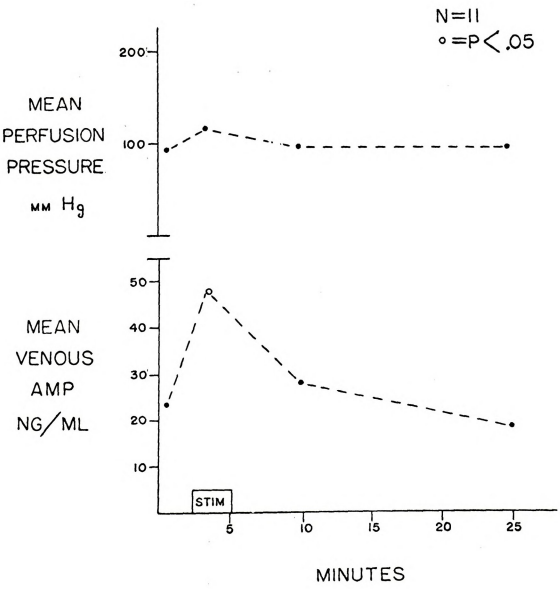

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The inflow and effluent oxygen partial pressure and pH were monitored in five experiments of this type. Mean inflow oxygen pressure was 597 mm Hg, whereas mean control outflow oxygen pressure was 369 mm Hg. During stimulation effluent oxygen pressure declined to a mean of 104 mm Hg. Fifteen minutes following stimulation mean oxygen pressure had risen to 292 mm Hg. Inflow pH was a mean of 7.41, whereas mean control effluent pH was 7.35. During stimulation mean effluent pH fell to 7.21. Mean post-stimulation pH at fifteen minutes was 7.11.

9. Effect of Isoproterenol on AMP Outflow

The vasodilator isoproterenol was infused intraarterially in eight experiments to determine whether or not dilation unaccompanied by muscular exercise would result in increased AMP efflux. Figure 14 shows that even though perfusion pressure decreased during the infusion period, AMP levels did not significantly change from control levels. Muscular exercise produced by gracilis nerve stimulation at 6V, 6 cps, 1.6 msec produced a significant increase in AMP efflux. Perfusion pressure increased in five of the eight experiments, and no change occurred in two others.

10. Effect of Nerve Stimulation on AMP Outflow during Gallamine Infusion

To determine whether the source of the increased AMP observed during nerve stimulation was nerve or muscle, the

Figure 14. Effect of isoproterenol and nerve stimulation on venous AMP. Following collection of two controls, isoproterenol was infused at 500 ng/minute into the gracilis muscle. At the first indication of vasodilation an effluent sample was collected, followed immediately by a second collection. Fifteen minutes after the end of the infusion a control was collected, followed by a five minute stimulation of the gracilis nerve at 6V, 6 cps, 1.6 msec. Two samples were collected during the stimulation. One final control was collected five minutes after the end of the stimulation. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of eleven experiments.

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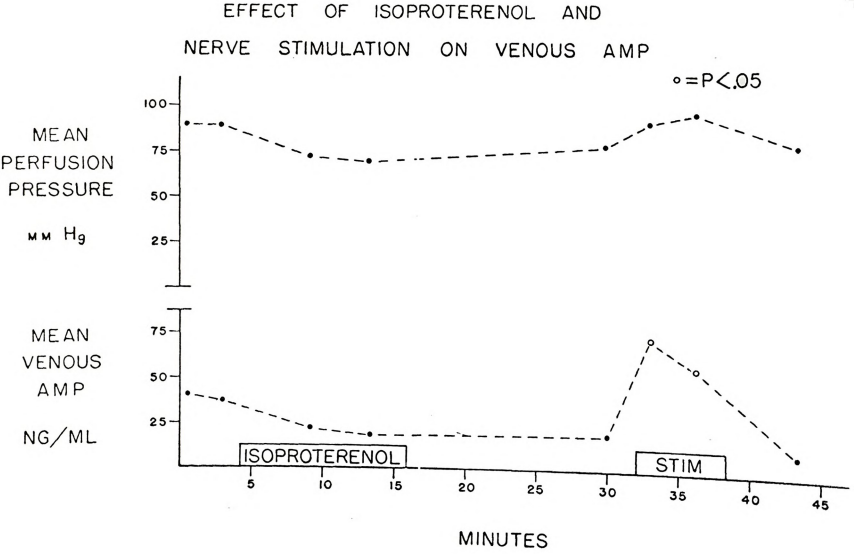

Figure 14

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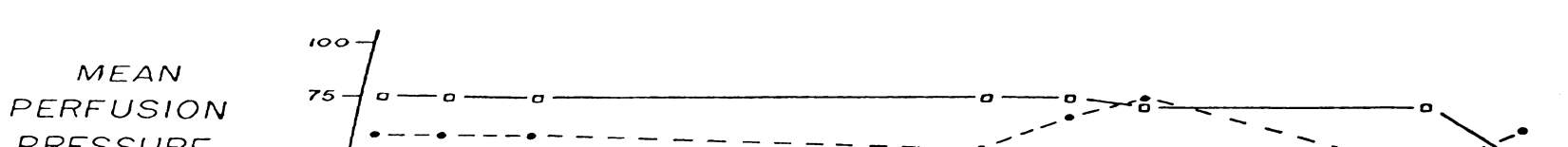
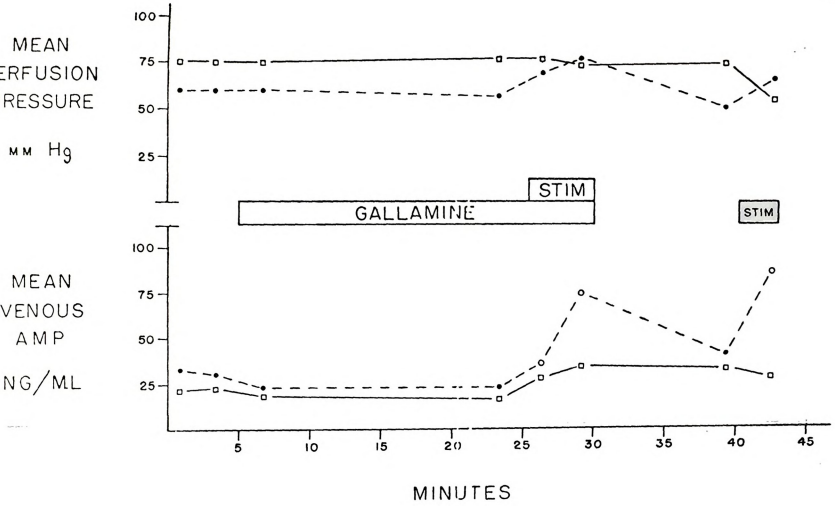
neuromuscular blocker gallamine was infused into the gracilis muscle. Figure 15 shows the results of two series of experiments in which this drug was used. In the first series (N = 7) the gracilis nerve was stimulated at 6V, 6 cps, 1.6 msec. Venous AMP did significantly increase above control levels during stimulation in this series, thereby indicating nerves as the source of the AMP since no muscular contraction occurred. However, perfusion pressure also increased during the stimulation, indicating vasoconstriction. Therefore, a second possibility for the source of the AMP existed in that it could have been released due to some effect of the vasoconstriction. The final stimulation without gallamine block produced the same results: increases in both effluent AMP and perfusion pressure.

In the second series of this type (N = 10) nerve stimulation was at 5V, 2 cps, 0.5 msec. Presumably sympathetic nerves would not be stimulated with these parameters. It can be seen from Figure 15 that in this case, although motor nerves were being stimulated, no significant increase in AMP outflow occurred. Likewise, perfusion pressure did not rise. The final stimulation without gallamine block also produced no increase in AMP efflux, even though skeletal muscle contraction was vigorous and marked vasodilation occurred, as seen from the decline in perfusion

Figure 15. Effect of nerve stimulation on venous AMP during gallamine infusion. Two series of experiments are shown in this figure. Solid circles represent the series in which nerve stimulation was at 6V, 6 cps, 1.6 msec. Open squares represent the series in which nerve stimulation was at 5V, 2 cps, 0.5 msec. Open circles represent significance at the .05 level. Experimental protocol was identical for both series. Two controls were collected, followed by infusion of 240 µg/minute of gallamine. A third control was collected just after the start of the infusion. Twenty minutes later a fourth control was collected. Then the gracilis nerve was stimulated for five minutes, during which time two samples were collected. Ten minutes after the end of the infusion a control was collected, followed by a two minute nerve stimulation. One sample was collected during this final stimulation. Each effluent sample collected was analyzed by the AMP assay. Each solid circle represents the mean of seven experiments. Each open square represents the mean of ten experiments.



during which time two samples were collected. At the end of the infusion a control was collected, followed by a two minute nerve stimulation. One sample was collected during this final stimulation. Each effluent sample collected was analyzed by the AMP assay. Each solid circle represents the mean of seven experiments. Each open square represents the mean of ten experiments.



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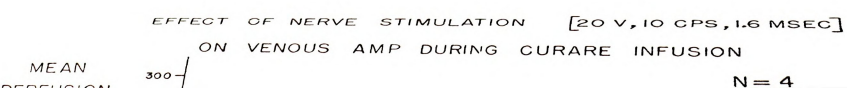
11. Effect of Nerve Stimulation on AMP Outflow during Curare Infusion

A group of experiments similar to the gallamine series was done in which curare was used as the neuromuscular blocker. The curare experiments were done both to further confirm the gallamine results and to determine if both drugs would produce similar results. Figure 16 shows that stimulation at 20V, 10 cps, 1.6 msec produces both vasoconstriction and a significant increase in AMP efflux. Again, no muscular contractions were produced. In Figure 17 it can be seen that stimulation at 5V, 2 cps, 1.6 msec produced no significant changes in AMP efflux. Likewise, perfusion pressure changed only slightly. These results reaffirm those of the gallamine series: (1) Muscular contraction does not appear to be necessary for increased AMP outflow to occur; (2) motor nerves do not appear to be the source of the AMP; and (3) sympathetic vasoconstriction is associated with increased AMP outflow from the artificially perfused gracilis muscle.

12. Effect of Nerve Stimulation at 5V, 2 cps, 0.5 msec on AMP Outflow

It was shown in the gallamine series at 5V, 2 cps, 0.5 msec that no increase in AMP outflow occurred during

Figure 16. Effect of nerve stimulation at 20V, 10 cps, 1.6 msec on venous AMP during curare infusion. Following collection of two controls, curare was infused at 30 µg/minute into the gracilis muscle. A third control was collected just after the start of the infusion. Twenty minutes later a fourth control was collected, followed by nerve stimulation for five minutes. Two samples were collected during the stimulation. Ten minutes after the end of the stimulation a final control was collected. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of four experiments.



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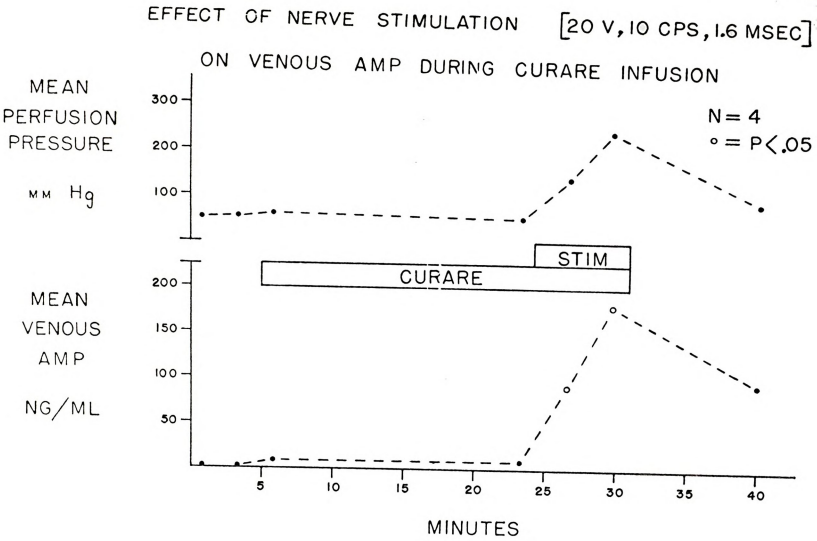

Figure 16

Figure 17. Effect of nerve stimulation at 5V, 2 cps, 1.6 msec on venous AMP during curare infusion. Following collection of two controls, curare was infused at 30 µg/minute into the gracilis muscle. A third control was collected just after the start of the infusion. Twenty-five minutes later a fourth control was collected, and then the gracilis nerve was stimulated for five minutes, during which time two samples were collected. Two final controls were collected approximately twenty minutes after the end of the stimulation. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of four experiments.

EFFECT OF NERVE STIMULATION [5V, 2/SEC, 1.6 MSEC]
ON VENOUS AMP DURING CURARE INFUSION

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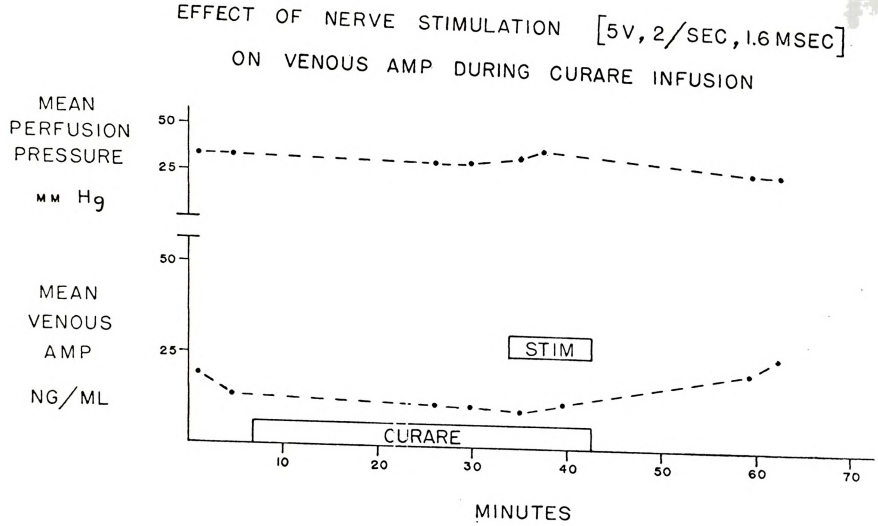

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the initial vasodilation which accompanied muscular exercise. It was still possible, however, that AMP could mediate the later stages of active hyperemia. Therefore, a series of six experiments was performed in which a twenty minute period of muscular exercise was produced by stimulating the gracilis nerve at 5V, 2 cps, and 0.5 msec. Figure 18 shows the results of this group of experiments. During the entire twenty minutes of muscular exercise, no significant increase in AMP outflow occurred. Muscle tension, excellent at first, gradually fell toward zero as the stimulation continued, indicating muscle fatigue and possible hypoxia. Perfusion pressure definitely decreased in three of the six experiments, indicating vasodilation. These results suggest that adenine nucleotides are not likely to play a role in the later stages of active hyperemia.

13. Effect of Nerve Stimulation at 22V, 10 cps, 1.6 msec on Venous AMP during Phentolamine Infusion

To further investigate whether the source of the observed AMP was nerve, the gracilis nerve was stimulated at high voltage after the alpha adrenergic receptors had been blocked with phentolamine. The results are shown in Figure 19. Perfusion pressure declined from a mean control value of 82 mm Hg to 50 mm Hg during the stimulation, indicating vasodilation. Venous AMP levels did not change

Figure 18. Effect of nerve stimulation at 5V, 2 cps, 0.5 msec on venous AMP. Following collection of two controls, the gracilis nerve was stimulated for twenty minutes. Four samples were collected at periodic intervals during the stimulation. Controls were collected immediately after the stimulation and ten minutes after the stimulation. Each effluent sample collected was analyzed by the AMP assay. Perfusion pressure and muscle tension were monitored throughout the experiments. Each point represents the mean of six experiments.

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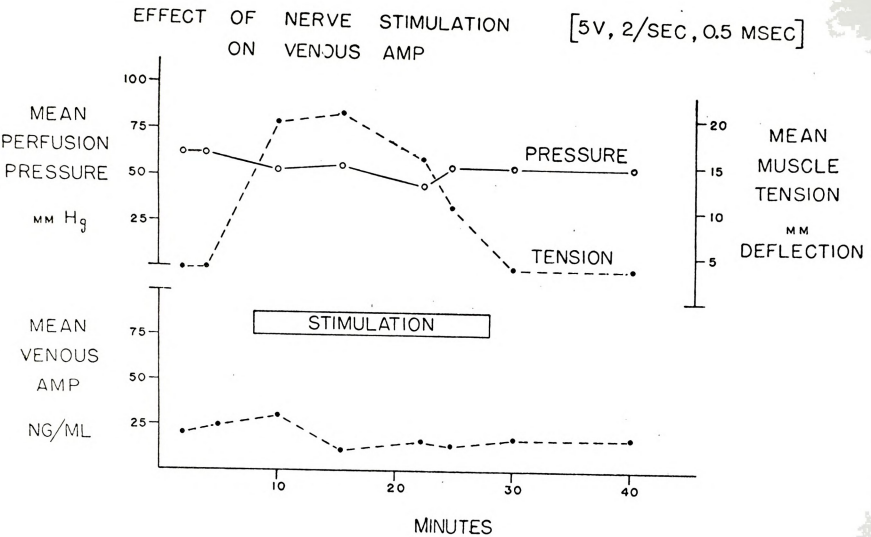
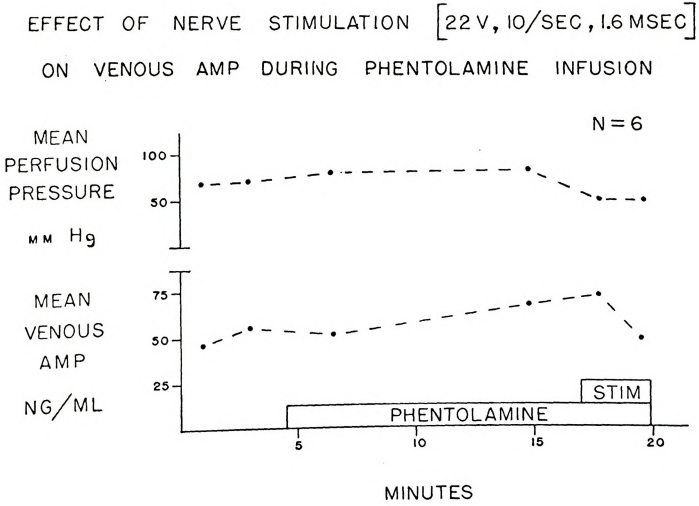


Figure 18

Figure 19. Effect of nerve stimulation at 22V, 10 cps, 1.6 msec on venous AMP during phentolamine infusion. Following collection of two controls, phentolamine was infused into the gracilis muscle at 50 µg/minute. A third control was collected just after the start of the infusion. Ten minutes later a fourth control was collected, followed by a three minute stimulation of the gracilis nerve. Two samples were collected during the stimulation. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of six experiments.

EFFECT OF NERVE STIMULATION [22 V, 10/SEC, 1.6 MSEC]
ON VENOUS AMP DURING PHENTOLAMINE

the AMP assay. Each point represents the mean of six experiments.



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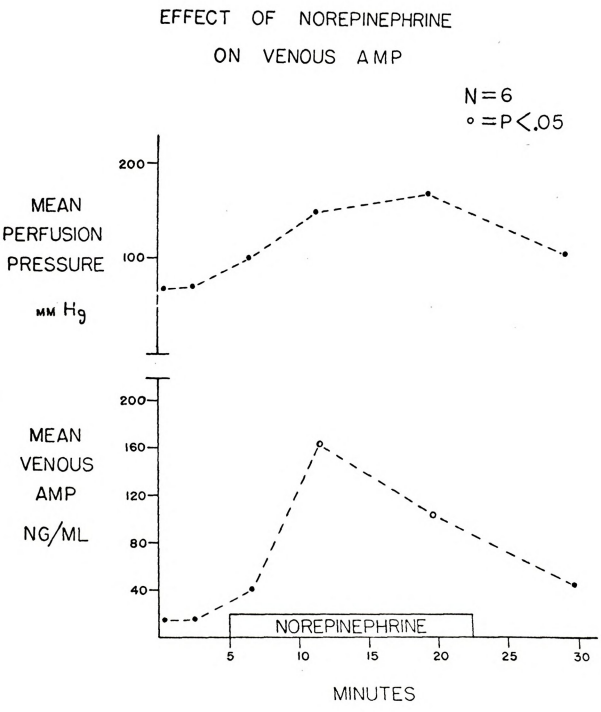
14. Effect of Norepinephrine on Venous AMP

The adrenergic transmitter norepinephrine was infused in a series of six experiments to determine if a vasoconstrictor agent could produce increased AMP outflow for an extended time period. The results are shown in Figure 20. During norepinephrine infusion perfusion pressure increased from a mean control value of 74 mm Hg to a maximum of 172 mm Hg. Venous AMP also significantly increased above control levels. The AMP level of the first sample collected after the start of the infusion was not significantly different from controls, although some increase was observed. This small increase can be explained by the fact that perfusion pressure had also risen only a small amount above control levels at this time. It can be seen that the third sample collected during the infusion shows a decline in AMP outflow. This is due to the fact that mean values have been plotted, and in one of the six experiments the AMP level was extremely high in the second sample collected. In the remaining five experiments AMP levels were fairly comparable between the second and third infusion samples, indicating that the AMP outflow can be maintained for at

Figure 20. Effect of norepinephrine on venous AMP. Following collection of two controls, norepinephrine was infused into the gracilis muscle at 760 ng/minute for eighteen minutes. Three samples were collected at periodic intervals during the infusion. One final control was collected ten minutes after the infusion was stopped. Each venous sample collected was analyzed by the AMP assay. Each point represents the mean of six experiments.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15. Effect of Norepinephrine on Venous AMP during Phentolamine Infusion

Phentolamine was infused prior to and during norepinephrine infusion in four experiments to determine if AMP outflow would be blocked along with vasoconstriction. The results are shown in Figure 21. It can be seen that norepinephrine produces no increased outflow of AMP when its vasoconstrictor action is blocked. This is in agreement with the results of phentolamine block of nerve stimulation.

16. Effect of Epinephrine at High Dose on Venous AMP

Figure 22 shows the results of infusion of 1.5 µg/minute of epinephrine (N = 5). This dosage produced a rise in perfusion pressure from a control mean of 65 mm Hg to 141 mm Hg. Venous AMP likewise significantly increased, from a mean control level of 57 ng/ml to mean infusion levels of 131 and 150 ng/ml. Phentolamine was then infused concurrently with the epinephrine and a sample collected at once. It can be seen that both venous AMP and perfusion pressure immediately fell to control levels. These results show that a second vasoconstrictor agent is associated with AMP outflow.

Figure 21. Effect of norepinephrine on venous AMP during phentolamine infusion. Following collection of two controls, phentolamine was infused into the gracilis muscle at 50 µg/minute for ten minutes. Two additional controls were collected at the beginning and the end of this period. Then norepinephrine, 760 ng/minute, was infused concurrently with the phentolamine for twelve minutes. Two samples were collected. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of four experiments.

EFFECT OF NOREPINEPHRINE ON VENOUS AMP
DURING PHENTOLOMINE INFUSION

EFFECT OF NOREPINEPHRINE ON VENOUS AMP
DURING PHENTOLOMINE INFUSION

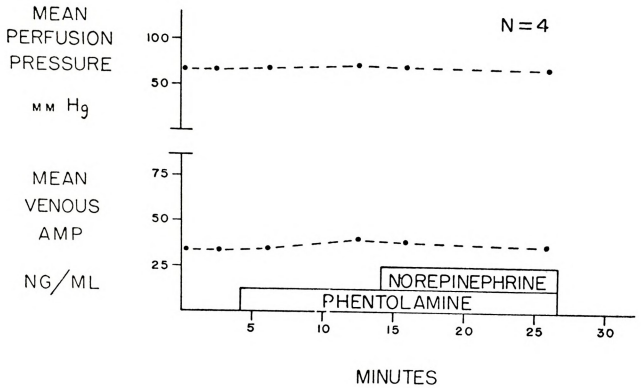

Figure 21

Figure 22. Effect of epinephrine at high dose on venous AMP. Following collection of two controls, epinephrine was infused intraarterially into the gracilis muscle at 1.5 µg/minute for ten minutes. Two samples were collected during this time. Phentolamine, 50 µg/minute, was then infused concurrently with the epinephrine for ten minutes. Two additional samples were collected. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of five experiments.

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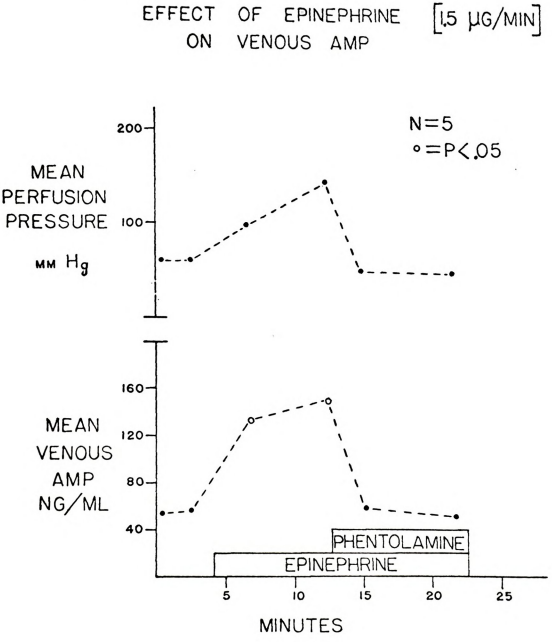

Figure 22

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17. Effect of Epinephrine at Low Dose on Venous AMP

Epinephrine was infused at 61 ng/minute, a dosage which was designed to be too low to produce vasoconstriction. These results are shown in Figure 23. Perfusion pressure remained at control levels throughout the infusion. Venous AMP also did not change, again suggesting the association between AMP outflow and vasoconstriction.

18. Effect of ADH on Venous AMP

To determine if a non-neurogenic vasoconstrictor agent would also be associated with AMP outflow, vasopressin was infused into the gracilis muscle in five experiments. The results are shown in Figure 24. Perfusion pressure rose during the infusion from mean control levels of 85 mm Hg to a maximum mean infusion level of 232 mm Hg. Mean venous AMP significantly increased above control levels during all three infusion samples. In the first two samples, the increase in AMP outflow paralleled the rise in perfusion pressure. However, in the third sample venous AMP declined somewhat, despite the continued increase in perfusion pressure. Perhaps this is due to the use of an artificially perfused preparation, where it might be difficult to maintain a supply of high-energy phosphate compounds if excessive demands were made on them. It could also indicate some type of a washout effect of adenine compounds from

Figure 23. Effect of epinephrine at low dose on venous AMP. Following collection of two controls, epinephrine was infused intraarterially into the gracilis muscle at 61 ng/minute for fifteen minutes. Three samples were collected at intervals during this infusion. One final control was collected ten minutes after the infusion was stopped. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of four experiments.

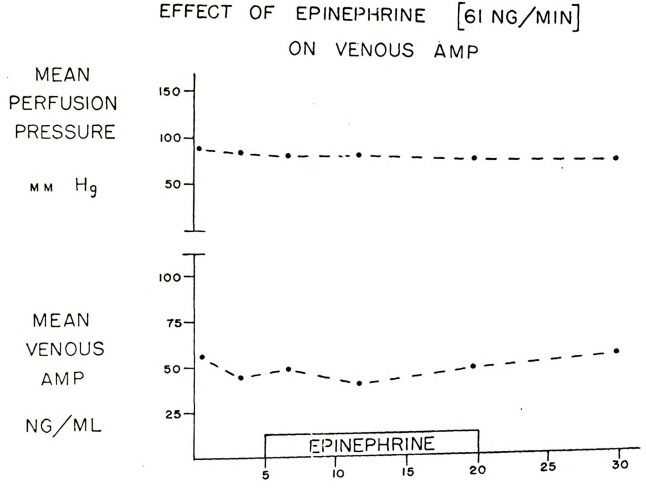
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Figure 23

Figure 24. Effect of ADH on venous AMP. Following collection of two controls, vasopressin was infused intraarterially into the gracilis muscle at .06 units per minute for seventeen minutes. Three samples were collected at intervals during this infusion. One final control was collected ten minutes after the end of the infusion. Each effluent sample collected was analyzed by the AMP assay. Each point represents the mean of five experiments.

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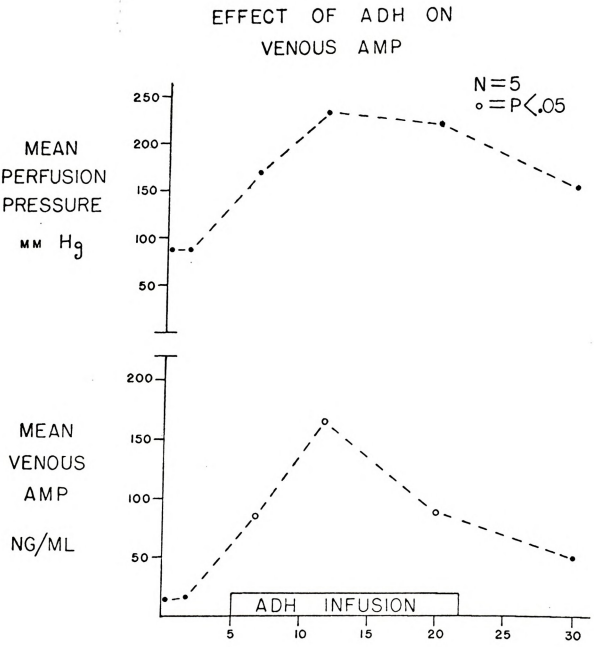

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cells which might still remain trapped in various blood vessels. Ten minutes after the infusion was stopped both venous AMP and perfusion pressure were returning to control levels.

19. Effect of Ouabain on Venous AMP

Only three experiments of this type were done. The results are shown in Table 6. No statistical analysis was performed, due to the small number of samples. It can be seen from Table 6 that no readily apparent relationship exists between AMP outflow and ouabain. Several doses of ouabain were used, and even the highest of these (225 µg/minute) had no major effect on either AMP or perfusion pressure. Presumably such a dosage would produce substantial depolarization. Perfusion pressure was steadily increasing, but very slowly. These limited results again indicate that vasoconstriction is a necessary prerequisite for increased AMP efflux.

20. Effect of Cells and Increased Collection Time on Venous AMP

Since cells contain a considerable quantity of adenine nucleotides, the possibility was investigated that blood cells and platelets were the source of the AMP observed in the samples. All samples were centrifuged before use in the AMP assay, and a qualitative estimate of the amount of cells centrifuged to the bottom of the centrifuge tube was then

Table 6. Effect of ouabain on venous AMP.

	Control	Control	$Ouabain_1$	$Ouabain_2$	$Ouabain_3$	$Ouabain_4$	Control
Mean Venous AMP	8	21	3	26	13	22	40
Mean Perfusion Pressure	65	65	67	70	75	77	80

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made for each sample. Figure 25 shows the results of plotting cells against venous AMP. It can be seen that no apparent relationship exists between amount of cells removed from the sample and venous AMP. For example, in some samples originally containing many cells, no AMP was measurable. In other samples where few, if any, cells were observed a considerable amount of AMP was measured. It is therefore unlikely that the AMP observed can be attributed to release from blood cells in the samples.

Another possibility considered was that the increases in AMP observed during vasoconstriction were due to the decreased venous effluent flow rate which often accompanied the vasoconstriction even though arterial flow was constant.* It was theorized that if a continual release of AMP into the effluent was occurring, then any decrease in the flow rate would increase the concentration of AMP in the effluent. However, no such relationship is evident in Figure 26. Substantial increases in venous AMP occurred with no increase in collection time. When increases in collection time did occur, the effect on venous AMP was not consistent. Therefore, it is unlikely that a decrease in flow rate can account for the increases in venous AMP observed during vasoconstriction.

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*The muscles often became very edematous at this time, and fluid leakage from sources such as cut vessels often occurred.

Figure 25. Effect of cells on venous AMP. Each sample used in the AMP assay was first centrifuged at 10,000 x G for five minutes at 0° C. A qualitative estimate of the amount of cells (or cell debris) in each sample was made immediately following centrifugation and the estimate recorded. Each point represents one sample. A = no cells; B = very few cells; C = few cells; D = moderate number of cells; E = many cells.

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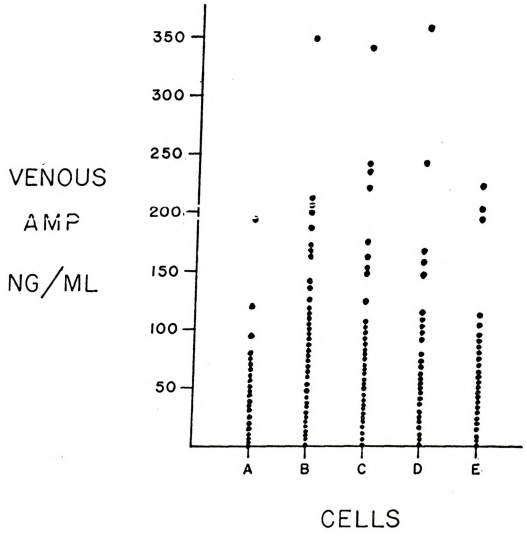
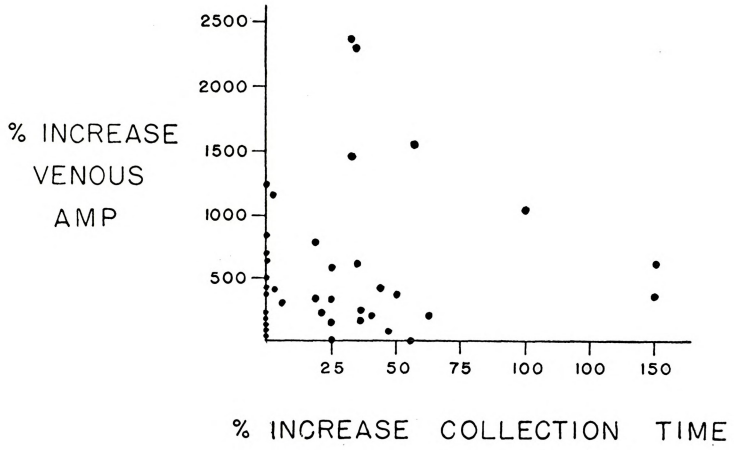


Figure 25

Figure 26. Effect of decreased flow rate on venous AMP. The amount of time required to collect a constant volume of effluent sample was determined for all experiments in which vasoconstriction occurred. The per cent increase in collection time during vasoconstriction as compared to control was then calculated and plotted against the corresponding per cent increase in venous AMP above control. Each point represents one experimental sample as compared to its control.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21. Relationship Between Perfusion Pressure and Venous AMP

Since the results of this thesis indicate that increases in perfusion due to a variety of causes were accompanied by increases in venous AMP, a linear regression analysis was performed on all the corresponding values for perfusion pressure and venous AMP listed in Appendix A. The correlation coefficient obtained from this analysis was 0.36, which indicates that perfusion pressure and venous AMP do not vary together. However, this analysis included data from many animals, each with its own characteristics. It does not seem unreasonable that absolute values for these factors might vary widely among animals. Within an animal, AMP did increase with perfusion pressure in most cases. Therefore, although the correlation coefficient does not show a relationship, one may still indeed exist.

DISCUSSION

Significance of the Observations Reported in this Thesis. The major conclusion of this thesis is that extracellular adenine nucleotides have no role in the promotion of active hyperemia. Neither ATP nor ADP could pass through the circulation of the gracilis muscle except when grossly unphysiological levels were intraarterially infused. The major breakdown product of both intraarterial ATP and ADP was venous AMP. When the venous effluent from the exercising muscle was analyzed for AMP when vasodilation or at least no vasoconstriction was present, no increases in AMP were found. This was true both at the onset of exercise and during a ten minute period of exercise. In most cases some vasodilation was observed. In addition, no increases in AMP occurred during the post-exercise period. The muscles were fatigued and hypoxic by the end of the ten minute exercise. Since the assay used in these studies was sensitive enough to detect 25 ng/ml of AMP, and since AMP could pass undegraded through the circulation of muscle with less than 10% uptake, and since no significant quantities of ATP were ever detected in the effluent experimental samples, it can be strongly suggested that extracellular

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adenine nucleotides do not mediate exercise hyperemia, at least in this preparation.

It must be considered, however, that it is not necessary for interstitial dilatory levels of AMP to be detectable in the venous effluent, since their site of production or release may be very close to their site of action. In this way dilatory concentrations could arise at specific sites within the tissue and yet not be detectable in the venous effluent. Also, the observed lack of venous AMP during skeletal muscle exercise described in this thesis may be caused by not having the gracilis muscle perfused with blood. It is conceivable that Ringer-perfused skeletal muscle is unable to produce extracellular AMP for promoting active hyperemia, whereas a blood perfused preparation can. The greater dilation during muscle contraction observed in the blood perfused hindlimb by Chen et al. (1972b) supports this possibility.

Criticism of Previous Reports of Adenine Nucleotide Release during Muscle Hyperemia. The results of passing ATP through the gracilis muscle vasculature show that it would be very difficult to detect or quantify low levels of ATP which might be released from within the gracilis muscle into the venous effluent during active or reactive hyperemia. With 10 μg/ml of ATP infused intraarterially, only 10 ng/ml of ATP could be detected in the venous effluent.

Chen et al. (1972b) and Forrester and Lind (1969) detected approximately 500 ng/ml of ATP in the venous effluent from exercising skeletal muscle. If one assumes that this ATP was released into the venous effluent within the muscle, enormous quantities of ATP would have had to have been released to account for this quantity of ATP in the effluent. Since this seems unlikely, a second possibility must be considered. The ATP observed could have come from the formed elements of the blood after collection. It should be mentioned that Chen et al. used stimulation parameters of 6V, 6 cps, 1.6 msec to produce muscular exercise. These parameters were associated with AMP release in the studies presented in the Results section of this thesis. If this AMP should have come from ATP, perhaps Chen would not have seen the increase in ATP had he stimulated at 5V, 2 cps, 1.6 msec, in which case sympathetic nerves are not stimulated. Both Forrester and Lind and Chen et al. collected their samples into EDTA. Since it has been shown that EDTA releases ATP from the formed elements of the blood as well as preventing ATP breakdown (Bockman et al., 1975b), any ATP found in control samples could be attributed to this source. However, both of these groups of investigators also found increased ATP levels during exercise. This increase is difficult to explain, since it would seem logical that EDTA would release ATP at the same rate in blood

from resting or exercising subjects. One point to consider here is the large variability of this method. The range of plasma ATP levels was from 0.17 to 1 nmole/ml when blood samples were incubated at 37° C for five minutes before adding EDTA and assaying for ATP (Bockman et al., 1975b). Such variation makes it very difficult to attempt to quantify ATP release during exercise.

Forrester (1972) still found increased ATP in the venous effluent with exercise even after switching from EDTA to citrate. Since samples were centrifuged for 25 minutes before the ATP assay and citrate affords little, if any, protection against the rapid breakdown of ATP which occurs in blood (Bockman et al., 1975b), it again seems possible that the observed increases could have been due to release from damaged blood cells. However, actual release of the ATP during muscle contractions can certainly not be ruled out in this case, particularly since no ATP was detectable in control samples treated by the same method. Lack of control sample ATP could have been due to lack of release from cells in the absence of EDTA or breakdown in the blood or both.

Parkinson (1973) also found increased venous plasma ATP with exercise. However, no details of the method for collecting the blood were given, so it is not possible to evaluate this study. Boyd and Forrester (1968) and

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Forrester and Hassan (1973) reported increased ATP in the artificial bathing medium of isolated, contracting frog sartorius muscles. For several reasons it is likely that this ATP came from damaged muscle cells. Boyd and Forrester suggested that this could not be the case, since no potassium was detected in the medium. However, leakage of as much as 10% of the total intracellular potassium may not have been detected by their flame photometric assay. This amount of leakage would only raise the extracellular potassium content by about 6%. Twenty to 40% increases in extracellular potassium were detected during exercise. The ATP increase amounted to only 1-2% of the intracellular stores and therefore could easily be accounted for by leakage from damaged muscle cells. In addition, the protein content of the bathing medium following 30 minutes of contraction rose to 3% of total muscle protein, suggesting muscle fiber damage. The small protein aldolase has been reported to be released from intact muscle fibers during exercise, but in amounts no greater than one per cent of total muscle aldolase (Zierler, 1957). Other factors which might have altered muscle cell membrane permeability were the lack of adequate oxygen and glucose in the artificial perfusate. Zierler reported that lack of either substance increases aldolase efflux from muscle by ten-fold.

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Dobson et al. (1971), in a more refined isolated, artificially perfused preparation, found no effluent ATP with exercise. Bockman et al. (1975b) confirmed this negative finding in the artificially perfused rat hindlimb. The perfusate was continuously bubbled with oxygen, and glucose was continuously supplied to the muscle. Their ATP assay, the firefly method, was very sensitive, capable of detecting nanogram levels of ATP.

Dobson et al. (1971) also detected no ATP during ischemic exercise in blood perfused limbs. Since no precautions were taken to prevent ATP breakdown, this could explain these negative findings. However, Bockman et al. (1975b) also found no consistent increases in plasma ATP when the blood was collected into EDTA, with either constant pressure or constant flow. Therefore, the evidence does not seem to indicate that ATP is the mediator of exercise hyperemia.

Furthermore, the results of the experiments with infusion of 1×10^{-9} M isoproterenol indicate that this compound probably does not dilate via AMP release into the extracellular fluid. Mayer and Stull (1971) found increases in rabbit muscle cyclic AMP upon injection of 4×10^{-10} moles of isoproterenol. Shepherd et al. (1973) found increased mesenteric blood flow upon infusion of either cyclic AMP or isoproterenol. Mesenteric artery cyclic AMP levels

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were increased by isoproterenol. It was suggested that cyclic AMP mediated the vasodilation produced by isoproterenol, possibly by altering membrane permeability to calcium or by inhibiting ATPase and thereby eliminating the energy production needed for contraction. It was not considered likely that the dilation was a result of the metabolic effects of isoproterenol or cyclic AMP. The possibility was also not considered likely that AMP was the actual mediator of the dilation. The breakdown of cyclic AMP to AMP catalyzed by phosphodiesterase would provide this AMP. However, a known inhibitor of phosphodiesterase, papaverine, is believed to dilate solely by increasing cyclic AMP levels (Kukovetz and Poch, 1970). The isoproterenol experiments in this thesis are in agreement with these studies.

Possible Role of Adenosine in Active Hyperemia. Studies by several groups indicate that both tissue and effluent adenosine levels increase during and after exercise (Bockman et al., 1975a; Tominaga et al., 1973d; Dobson et al., 1971; Tabaie et al., 1975). In the studies by Tominaga's group AMP and adenosine could not be distinguished from each other with their assay. Therefore, based on the results of this thesis, it is likely that the increase occurred in adenosine and not AMP during exercise. This same reasoning applies to the results of bioassay studies, where venous blood from exercising muscle was

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constrictor in the kidney and dilator in the limb (Scott et al., 1965), suggesting either adenosine or AMP being the mediator of active hyperemia. The studies of this thesis indicate that adenosine is the more likely compound to have produced the renal constriction and skeletal muscle dilation.

Further evidence that adenosine may mediate skeletal muscle active hyperemia was provided by Tabaie et al. (1975). Theophylline, a competitive inhibitor of adenosine, was infused into the canine gracilis muscle. Both exercise dilation and the dilatory response to 10 γ adenosine injection were significantly reduced during this infusion. The evidence presented by Rubio et al. (1973) also supports a role for adenosine in producing dilation, since they localized 5'-nucleotidase activity in regions very near the resistance vessels. Adenosine could conceivably be the mediator of dilation induced by hypoxia, although no clear-cut evidence exists to substantiate this theory. The changes in many substances which occur during exercise and the increased breakdown of ATP which also occurs could raise intracellular AMP levels. Depression of deaminases which is known to occur (Watanabe et al., 1973) could result in increased adenosine levels, which could in turn cause adenosine to diffuse out of the cells and produce dilation. Since a gradient should be assumed for venous adenosine

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levels to appear, tissue adenosine levels would be higher. Considering this plus the large increases observed in venous adenosine breakdown products, inosine and hypoxanthine, it seems probably that adenosine may play a role in mediating exercise hyperemia, and in particular, the hyperemia of sustained exercise.

Possible Role of Extracellular AMP in Skeletal Muscle Reactive Hyperemia. The effect of occlusion on venous AMP was consistent with the findings of others in skeletal muscle. AMP levels only increased following occlusion in about one-half of the experiments. AMP increases and dilation did not correlate, as dilation often occurred with no AMP increase and AMP increases occurred without dilation (see Appendix, Table 1). The increases in AMP which did occur may have resulted from the opening of previously closed vessels within the muscle. Burton and Johnson (1972) found that normally only about 30% of the increased flow of reactive hyperemia was due to the opening of new capillaries. The rest of the increase occurred via increased flow in previously open vessels. Since the muscles used in the studies presented in this thesis were artificially perfused, the vasculature was in many cases fairly well dilated. Perhaps increased variability in the number of closed capillaries which opened during occlusion in this preparation can account for the inconsistent pattern of AMP

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increases observed. The renal vasoconstrictor activity of the blood from an occluded hindlimb may have been due to adenosine (Tominaga et al., 1973a). The results of this thesis suggest that adenine nucleotides do not mediate reactive hyperemia.

A comparison of the effect of flow rate on AMP uptake showed that considerably more AMP was taken up at slow flow rates than at high flow rates. Since flow rates are high during active hyperemia, these results indicate that more AMP might appear in the venous outflow. Of course, the dilution effect of the increased blood flow upon venous AMP would tend to offset this. No definite conclusions can be drawn regarding the effect of flow rate on AMP breakdown due to the small number of samples and the inconsistent results.

Vasoconstriction as a Possible Cause of Increased Venous AMP. The results of this thesis demonstrate that increased levels of AMP appear in the venous effluent during vasoconstriction produced by sympathetic nerve stimulation, norepinephrine, epinephrine, and vasopressin. The vasoconstriction appears to be the stimulus for the AMP outflow, since low doses of epinephrine and alpha adrenergic blockade of the constrictor effects of sympathetic nerve stimulation and the catecholamines were not accompanied by increased AMP outflow. Also, although all these agents may

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constrict via the same mechanism, it is more likely that two or more different mechanisms of constriction may be involved. This would lend support to the theory that the vasoconstriction, not the vasoconstrictor, produces the AMP outflow.

The source of this AMP could be skeletal muscle, nerve, vascular smooth muscle, or the formed elements of the blood. Contraction of skeletal muscle is not a cause of the increased venous AMP, since vigorous muscle contractions can occur with no concomitant efflux of AMP and increased AMP efflux can occur with no skeletal muscle contractions. Neuron stimulation is also an unlikely cause of the AMP release. Alpha motor nerve stimulation alone produced no increase in AMP outflow, as shown by the low voltage stimulation experiments. Sympathetic nerve stimulation was associated with increased AMP efflux; however, vasoconstriction also resulted from the stimulation. When the vasoconstriction was blocked with phentolamine, the increased AMP efflux was also blocked upon sympathetic nerve stimulation. Intrinsic nerves, as suggested by Honig and Frierson (1974), are also a possible source of the AMP. However, very little is known concerning these intrinsic nerves, and it is thus impossible to predict if phentolamine blocks their stimulation in the studies reported in this thesis.

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during vasoconstriction. In the preparation used in this thesis an attempt was made to wash out all formed elements before starting the experiment and to centrifuge them out of the samples. Pre-experimental muscular exercise was produced by nerve stimulation, and the muscle was then perfused for 20 minutes before starting the experiment. However, some cells were usually visible upon centrifugation in most samples.* In most cases it was not possible to completely wash all the blood cells out of the muscle for unknown reasons. Therefore, these residual formed elements could have caused the elevated AMP levels associated with vasoconstriction. It is unlikely that erythrocytes were the source of the AMP, since no obvious relationship was observed between sample erythrocytes observed and AMP concentration, as shown in Figure 25. In addition, ATP concentration was not significant in the samples. If formed elements release artifactual AMP into the samples, one would have expected high ATP levels as well, since cells contain much more ATP than AMP and since ATP does not break down in venous effluent samples, although it would if released within the muscle vasculature.

Groom et al. (1973) also observed difficulty in washing out blood cells from muscle. In the cat gastrocnemius

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*No specific cell counts were made. Only a qualitative estimate of the amount of cells present in each sample after centrifugation was made.

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muscle they found four different patterns of blood flow within a resting muscle. For three of these, washout was rapid. The fourth had a washout half-time of 47 minutes and was found to consist entirely of reticulocyte washout. It was suggested that reticulocytes adhere to vessel walls much more than other blood cells, thereby accounting for their slow washout. Vasodilators, such as acetylcholine, did not increase reticulocyte clearance, indicating that cellular rather than vascular factors determine clearance in this case.

The study by Groom et al. further demonstrates the non-homogenous perfusion of skeletal muscle which normally occurs. Other workers also have evidence to support this finding. Studies by Moore and Baker (1971), using ^{51}Cr-labelled cells and ^{131}I-labelled protein, suggested the presence in muscle of poorly perfused parallel circuits. Renkin and Rosell (1962) concluded from studies of ^{86}Rb extraction in muscle that stimulation of sympathetic vaso-constrictor nerves decreased homogeneity of blood perfusion. Pappenheimer (1941b) suggested that sympathetic stimulation diverted blood flow through arteriovenous anastomoses. Ballard et al. (1964) calculated that 40% of the resting flow to skeletal muscle passes through non-nutritive shunt vessels. Friedman (1965), observing rubidium extraction, concluded that total muscle blood flow is greater than

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capillary blood flow. Nakamura et al. (1972) confirmed this finding. Walder (1968) suggested that although there are several circulatory paths in skeletal muscle, all are nutritive. Presumably, one path is for connective tissue nutrition and the other two are for muscle fiber nutrition. Therefore, variations in the number of perfused and non-perfused circuits could have contributed to the variability in the amount of cells observed in the studies of this thesis.

Eriksson and Lisander (1972) studied the effect of sympathetic stimulation on blood-perfused muscles of cats. Vessels smaller than 10 μ showed no diameter changes in response to the stimulation; maximal diameter reduction occurred in vessels at the 30 μ size. With intense sympathetic stimulation, periodic "breakthroughs" of about 30 seconds duration occurred. Leucocytosis was also observed, a factor which as a remote possibility could have produced the increased AMP outflow noted in this thesis.

In addition to reticulocytes and leucocytes, platelets could also be the source of the AMP observed. Tangen et al. (1973) found leakage of ATP and ADP from platelets when they were transferred to a non-protein medium. Addition of albumin to the medium prevented the efflux. Since a non-protein artificial perfusate was used in all of the studies presented in Results, any residual platelets could have

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leaked nucleotides. This could account for any AMP levels observed in control samples; the variability of control AMP levels could therefore be due to varying amounts of residual platelets between preparations. However, it is not clear why such leakage would increase only with vasoconstriction. Another factor which suggests that formed elements of the blood are not the source of the AMP is the immediate return of AMP to control levels upon phentolamine block of vasoconstriction. If the constriction in some way damaged the blood cells to cause the AMP release, it should continue for a time in the absence of the constriction.

The use of a non-protein artificial perfusate resulted in significant edema formation in most experiments. Muscle weight often doubled. Therefore, it should be considered as to whether edema could in some way give rise to the observed increases in AMP. Groom et al. (1973) found no difference in red cell clearance between edematous and non-edematous muscles. However, Paradise et al. (1971) found a greater degree of perfusion heterogeneity in surgically isolated as opposed to unisolated muscles. The perfusion heterogeneity was related to edema formation, which was greater in isolated muscles. This perfusion heterogeneity resulting from edema could cause stagnation of cells trapped in unperfused vessels, which in turn could be the source of the AMP observed with vasoconstriction. However, it is

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considered unlikely that edema actually was responsible for the AMP outflow, since in edematous muscles at rest or contracting to the point of fatigue no AMP outflow occurred unless vasoconstriction also occurred.

Vascular smooth muscle is also a likely source of the AMP. During vasoconstriction the smooth muscle cells contract, a process which requires ATP breakdown. With strong or prolonged contraction AMP levels could build up and then leave the cells, thereby producing the observed efflux. Studies utilizing radioactive tracers in isolated strips of arterioles might determine if this theory is correct. Whatever the source of the AMP, its efflux during vasoconstriction seems to be a real phenomenon, since it occurs in response to different constrictor agents and in muscles with little edema as well as in very edematous muscles. It seems likely that this response to vasoconstriction may also occur in intact, blood perfused muscles.

Another possible source of the observed AMP increase with vasoconstriction is cyclic AMP. The vasoconstrictor agents used (norepinephrine, epinephrine, and ADH) are known to stimulate cyclic AMP formation in various tissues, and sympathetic nerves release norepinephrine as their chemical transmitter. Any cyclic AMP formed could be broken down to AMP by phosphodiesterase; the AMP could then diffuse out of the cells and appear in the effluent.

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This possibility is considered unlikely, however, since intraarterial isoproterenol, which stimulates cyclic AMP formation, does not result in increased effluent levels of AMP.

Should this observation be real, the physiological significance of AMP release with vasoconstriction seems obscure. One possibility is that the AMP release represents a mechanism to counteract vasoconstriction and maintain tissue blood flow. Such a response might be important in situations of strong sympathetic stimulation, such as hemorrhagic shock. Haddy et al. (1965) noted that the active constriction of precapillary vessels in the dog forelimb following hemorrhage was not well maintained. The mechanism of the constriction's waning was not determined. Bond et al. (1967) reported a progressive increase in skeletal muscle vascular conductance in irreversible shock. Lundgren et al. (1964) found that the waning response to vasoconstriction was more pronounced in the pre- than in the post-capillary vessels.

LePage (1946) and Chaudry et al. (1972-73) found depleted ATP stores in muscle tissue of rats in shock. Threlfall and Stoner (1957) found increased muscle levels of AMP in the rat after two and six hours of ischemia but not after four hours of ischemia. However, Chaudry et al. (1974) found decreased tissue AMP levels two hours after

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hemorrhage in rats. Talaat et al. (1964) reported that ATP administration increased the survival rate of dogs in shock. However, Sharma and Eiseman (1966) found that AMP administration did not protect against shock nearly as well as ATP administration. Chaudry et al. (1975a) found no change during shock in the activity of the enzymes located on the muscle cell surface which break down adenine nucleotides. Therefore, it is possible that AMP outflow counteracts the decreased blood flow resulting from the constriction. Indeed, such a response was seen transiently in many of the strong stimulation experiments. A transient (approximately 20 second) decrease in the perfusion pressure appeared soon after the initial constriction.

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SUMMARY AND CONCLUSIONS

The role of adenine nucleotides in active hyperemia and post-occlusion flow was studied in the isolated, artificially perfused, canine gracilis muscle. ATP and ADP were not able to pass undegraded through the gracilis muscle vasculature except when infused in large quantities (10 μg/ml or greater). ATP and ADP were degraded almost entirely to AMP on a single pass through the muscle vasculature. Almost all intraarterial AMP was able to pass through the muscle vasculature without breakdown. Therefore, to determine whether or not AMP is released during muscle exercise and after occlusion, an assay was set up to analyze for nanogram levels of AMP.

During post-occlusion increased flow, no consistent increases in AMP were found in the venous effluent. During active hyperemia induced by stimulation of the gracilis nerve at 5V, 2 cps, 1.6 msec no significant increases in venous AMP were found. This was true at the start of contractions, during a ten minute period of fatiguing exercise, and in the post-contraction period. The vasodilator isoproterenol did not increase venous AMP outflow. Vasoconstriction produced by gracilis nerve stimulation at 6V,

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6 cps, 1.6 msec or at 20V, 10 cps, 1.6 msec was associated with significant increases in AMP efflux. This was true even when muscle contraction was blocked with either gallamine or curare. Epinephrine, norepinephrine, and vasopressin infused intraarterially also produced vasoconstriction and increased AMP outflow. Both the vasoconstriction and the AMP outflow were prevented by phentolamine block of norepinephrine, epinephrine, and nerve stimulation.

Therefore, it is unlikely that extracellular adenine nucleotides mediate active hyperemia or post-occlusion flow changes in this preparation. Isoproterenol probably does not dilate via release of AMP. Since vasoconstrictions produced by nerve stimulation, norepinephrine, epinephrine, and vasopressin all increase AMP outflow from the gracilis muscle, this AMP outflow may antagonize the vasoconstriction and thereby attempt to maintain tissue blood flow. The source of the AMP was not determined but could be nerve, formed elements of the blood, vascular smooth muscle, or any other cells within the tissue, such as connective tissue cells.

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APPENDIX

Table 1. *Effect of occlusion and nerve stimulation on gracilis muscle venous AMP.* (*Results*, Figure 12)
Key: C = control; R = post-occlusion flow; S = nerve stimulation, 6V,

Table 1. Effect of occlusion and nerve stimulation on gracilis muscle venous AMP. (Results, Figure 12)
Key: C = control; R = post-occlusion flow; S = nerve stimulation, 6V, 6 cps, 1.6 msec.

	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
Dog #	C	C	R_1	R_2	C	S_1	S_2	C	C	C	R_1	R_2	C	S_1	S_2	C
1	15	20	18	16	0	28	17	18	75	75	75	75	65	50	45	50
2	2	30	29	20	14	18	10	0	35	35	35	35	35	30	30	20
3	38	45	93	43	35	55	33	5	120	125	120	120	105	90	80	75
4	15	33	28	32	36	60	28	50	35	40	35	35	25	40	40	20
5	28	15	50	20	46	41	85	50	20	20	20	20	50	45	45	20
6	48	45	38	38	45	63	61	37	90	90	45	85	90	35	30	30
7	13	12	15	25	38	45	26	14	35	35	30	35	40	10	10	15
8	0	5	0	0	0	0	0	0	145	170	150	140	60	60	70	35
9	0	25	75	0	0	0	0	0	95	95	80	90	95	80	70	95
Mean	18	26	38	22	24	34	29	19	72	76	66	71	63	49	47	40
S.E.	6	5	10	5	7	8	9	7	15	17	15	14	10	8	8	9

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Table 2. Effect of nerve stimulation (6V, 6 cps, 1.6 msec) on gracilis muscle venous AMP. (Results, Figure 13)
Key: C = control; S = nerve stimulation

	Venous AMP, ng/ml				Perfusion Pressure, mm Hg			
Dog #	C	S	C	C	C	S	C	C
1	26	44	66	-	95	150	-	-
2	14	31	-	23	125	175	-	125
3	23	70	-	18	80	100	-	75
4	8	26	23	0	75	100	100	100
5	13	38	13	17	100	135	110	95
6	14	7	3	7	100	100	95	100
7	28	38	18	8	80	100	80	60
8	8	18	14	16	150	150	150	150
9	18	48	10	12	75	60	60	60
10	61	95	54	57	85	85	95	100
11	46	115	51	35	95	135	100	95
Mean	24	48	28	19	96	117	99	96
S.E.	5	10	8	5	7	10	9	9

Table 3. Effect of intraarterial infusion of isoproterenol on gracilis muscle venous AMP. (Results, Figure 14)
Key: C = control; I = isoproterenol; S = nerve stimulation, 6V, 6 cps,

Table 3. Effect of intraarterial infusion of isoproterenol on gracilis muscle venous AMP. (Results, Figure 14)
Key: C = control; I = isoproterenol; S = nerve stimulation, 6V, 6 cps, 1.6 msec.

Dog #	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
	C	C	I_1	I_2	C	S_1	S_2	C	C	C	I_1	I_2	C	S_1	S_2	C
1	83	90	187	73	106	340	200	67	110	110	60	50	85	80	75	80
2	0	0	0	0	7	65	42	26	50	50	45	45	50	50	55	50
3	7	27	18	0	0	53	43	0	85	85	85	80	100	105	105	85
4	2	0	0	0	0	0	0	0	75	75	75	75	75	75	75	70
5	208	167	97	66	18	94	78	0	130	135	135	135	125	135	125	125
6	5	3	44	0	0	9	8	2	100	100	50	50	60	100	135	70
7	16	8	0	0	0	5	-	7	55	55	40	40	40	40	40	40
8	0	0	0	0	0	7	22	0	100	100	85	80	90	125	140	100
Mean	40	37	43	17	16	72	56	13	88	89	72	69	78	89	94	78
S.E.	26	22	24	11	13	40	26	8	10	10	11	11	10	12	13	10

Table 4. *Effect of nerve stimulation (5V, 2 cps, 0.5 msec) on venous AMP during Gallamine infusion.* (Results, Figure 15)
Key: C = control; S = stimulation; G = gallamine

Table 4. Effect of nerve stimulation (5V, 2 cps, 0.5 msec) on venous AMP during Gallamine infusion. (Results, Figure 15)
Key: C = control; S = stimulation; G = gallamine

Dog #	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
	C	C	C+G	C+G	S_1	S_2	C	S_3	C	C	C+G	C+G	S_1	S_2	C	S_3
1	63	62	50	45	38	98	70	64	120	120	115	130	125	120	110	75
2	30	40	25	47	125	40	39	35	35	35	35	60	60	60	75	60
3	20	33	25	41	38	53	62	22	30	30	30	30	30	35	35	25
4	22	40	26	5	13	76	59	35	45	45	45	40	35	35	35	30
5	0	0	23	0	0	0	0	12	35	35	35	35	35	35	20	15
6	70	45	50	45	43	62	55	68	110	110	115	100	100	100	95	65
7	0	0	0	0	0	0	0	10	45	45	45	45	50	50	55	45
8	5	4	0	0	0	0	0	0	60	60	55	50	50	55	40	35
9	0	0	0	0	0	0	20	19	70	70	70	75	70	70	70	55
10	0	0	0	0	0	0	12	12	200	200	210	215	215	215	215	210
Mean	21	22	20	18	26	33	32	28	75	75	76	78	77	78	75	62
S.E.	8	8	6	7	12	33	9	7	17	17	18	18	18	18	18	18

Table 5. *Effect of nerve stimulation (6V, 6 cps, 1.6 msec) on venous AMP during gallamine infusion.* (Results, Figure 15)

Table 5. Effect of nerve stimulation (6V, 6 cps, 1.6 msec) on venous AMP during gallamine infusion. (Results, Figure 15)
Key: C = control; S = stimulation; G = gallamine

Dog #	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
	C	C	C+G	C+G	S_1	S_2	C	S_3	C	C	C+G	C+G	S_1	S_2	C	S_3
1	64	53	48	38	75	220	120	167	60	60	50	45	60	70	75	85
2	97	67	45	28	43	87	25	75	10	10	10	10	20	20	10	10
3	18	32	22	42	35	54	-	-	25	25	25	25	30	30	25	20
4	50	49	47	49	53	73	57	80	75	75	75	75	80	95	80	70
5	0	0	0	0	0	5	0	0	75	75	80	60	70	75	45	35
6	0	0	0	0	5	20	8	28	90	90	90	115	125	130	75	85
7	0	0	0	0	33	70	30	165	90	90	90	60	90	115	50	150
Mean	33	29	23	22	35	76	40	86	61	61	60	56	68	76	51	65
S.E.	15	11	9	8	10	27	18	28	12	12	12	13	14	16	10	18

Table 6. *Effect of nerve stimulation (20V, 10 cps, 1.6 msec) on gracilis muscle venous AMP during curare infusion.* (Results, Figure 16)

Table 6. Effect of nerve stimulation (20V, 10 cps, 1.6 msec) on gracilis muscle venous AMP during curare infusion. (Results, Figure 16)
Key: C = control; CC = control plus curare; S = nerve stimulation plus curare

Dog #	Venous AMP, ng/ml							Perfusion Pressure, mm Hg						
	C	C	CC	CC	S_1	S_2	C	C	C	CC	CC	S_1	S_2	C
1	0	0	0	0	158	243	190	30	30	30	30	150	250	80
2	0	0	40	0	83	243	58	45	45	55	45	150	250	55
3	0	0	0	13	70	145	78	70	70	70	60	150	250	100
4	0	0	0	22	45	93	63	60	60	65	65	100	200	95
Mean	0	0	10	9	89	181	97	51	51	55	50	138	238	83
S.E.	0	0	10	5	24	37	31	9	9	9	8	13	13	10

Fig. 5. ENG at 45 percent stimulation (SV, 2 cps, 1.6 msec) on gracilis muscle

Table 7. Effect of nerve stimulation (5V, 2 cps, 1.6 msec) on gracilis muscle venous AMP during curare infusion. (Results, Figure 17)
Key: C = control; CC = control plus curare; S = nerve stimulation plus curare

Dog #	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
	C	C	CC	CC	S_1	S_2	C	C	C	C	CC	CC	S_1	S_2	C	C
1	66	50	33	40	35	40	42	34	25	25	40	40	40	40	40	40
2	0	0	0	0	0	0	0	0	50	50	30	30	35	50	25	25
3	0	0	0	0	0	0	33	45	50	50	40	40	50	55	40	40
4	8	0	10	5	6	8	8	25	10	10	10	10	10	10	10	10
Mean	19	13	11	11	10	12	21	26	34	34	30	30	34	39	29	29
S.E.	16	13	8	10	8	10	10	10	10	10	7	7	9	10	7	7

Table 8. *Effect of nerve stimulation (5V, 2 cps, 0.5 msec) on gracilis muscle venous AMP.* (Results, Figure 18)

Table 8. Effect of nerve stimulation (5V, 2 cps, 0.5 msec) on gracilis muscle venous AMP. (Results, Figure 18)
Key: C = control; S = nerve stimulation

Dog #	Venous AMP, ng/ml								Perfusion Pressure, mm Hg							
	C	C	S_1	S_2	S_3	S_4	C	C	C	C	S_1	S_2	S_3	S_4	C	C
1	0	0	0	0	0	0	0	0	55	55	40	35	35	35	35	35
2	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-
3	38	38	43	30	31	29	27	33	85	85	90	85	85	85	85	85
4	0	25	18	18	25	25	38	28	45	45	45	45	45	45	45	45
5	63	50	65	-	33	-	-	-	60	60	50	-	50	-	-	-
6	25	35	30	-	0	-	-	-	65	65	35	-	20	-	-	-
Mean	21	25	28	12	15	14	16	15	62	62	52	55	47	55	55	55
S.E.	11	9	10	7	7	8	10	9	7	7	10	15	11	15	15	15

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Table 9. Effect of nerve stimulation (5V, 2 cps, 0.5 msec) on gracilis muscle tension. (Results, Figure 18)
Key: C = control; S = nerve stimulation

Dog #	Average Muscle Tension, mm Deflection							
	C	C	S_1	S_2	S_3	S_4	C	C
1	0	0	17	17	11	8	0	0
2	-	-	-	-	-	-	-	-
3	0	0	21	35	33	10	0	0
4	0	0	18	14	6	2	0	0
5	0	0	15	-	6	-	-	-
6	0	0	8	-	4	-	-	-
Mean	0	0	16	17	12	7	0	0

Table 10. Effect of nerve stimulation (22V, 10 cps, 1.6 msec) on venous AMP

Table 10. Effect of nerve stimulation (22V, 10 cps, 1.6 msec) on venous AMP during phentolamine infusion. (Results, Figure 19)
Key: C = control; S = stimulation; P = phentolamine

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	C+P	C+P	S_1	S_2	C	C	C+P	C+P	S_1	S_2
1	38	73	-	93	75	68	60	65	100	100	50	50
2	30	28	18	29	35	33	70	70	70	70	70	70
3	78	70	105	105	118	70	70	75	85	100	40	40
4	33	45	30	48	40	35	55	55	50	50	45	45
5	58	58	55	60	88	56	75	75	80	90	55	55
6	38	60	50	-	74	31	70	75	85	-	40	40
Mean	46	56	52	67	72	49	68	69	78	82	50	50
S.E.	8	7	15	14	13	7	3	3	7	10	5	5

Table 11. *Effect of norepinephrine on venous AMP.* (Results, Figure 20)
Key: C = control; N

Table 11. Effect of norepinephrine on venous AMP. (Results, Figure 20)
Key: C = control; N = norepinephrine

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	N_1	N_2	N_3	C	C	C	N_1	N_2	N_3	C
1	40	41	100	525	118	98	75	80	110	150	145	110
2	0	0	0	43	63	0	50	50	75	100	125	80
3	0	0	5	48	73	30	70	70	100	165	175	110
4	50	43	98	138	110	64	95	95	130	180	190	125
5	0	0	0	125	164	45	80	80	100	175	230	135
6	3	12	38	90	105	33	70	70	90	130	170	75
Mean	16	16	40	162	106	45	73	74	101	150	173	106
S.E.	9	8	20	74	15	14	6	6	8	12	15	10

Effect of norepinephrine on venous AMP during phentolamine infusion.

Table 12. Effect of norepinephrine on venous AMP during phentolamine infusion. (Results, Figure 21)
Key: C = control; P = phentolamine; N = norepinephrine

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	C+P	C+P	N+P	N+P	C	C	C+P	C+P	N+P	N+P
1	43	48	60	73	68	59	75	75	80	90	90	90
2	38	25	35	43	34	23	45	45	45	45	45	40
3	0	0	0	18	7	12	50	50	50	50	50	50
4	50	55	43	48	50	53	100	100	100	100	100	100
Mean	33	32	35	46	40	37	68	68	69	71	71	70
S.E.	11	12	13	11	13	11	13	13	13	14	14	15

Table 13. *Effect of intraarterial infusion (1.5 µg/min.) of epinephrine on gracilis muscle venous AMP.* (Results Pigmy 01)

Table 13. Effect of intraarterial infusion (1.5 μg/min.) of epinephrine on gracilis muscle venous AMP. (Results, Figure 22)
Key: C = control; E = epinephrine; P = phentolamine

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	E	E	E+P	E+P	C	C	E	E	E+P	E+P
1	40	55	45	78	41	56	50	50	60	70	50	45
2	70	60	203	223	83	50	85	85	125	165	60	55
3	60	60	98	138	50	73	60	65	100	185	35	35
4	33	38	193	150	53	32	75	75	125	185	65	60
5	56	74	115	163	58	39	50	50	70	100	30	30
Mean	52	57	131	150	57	50	64	65	96	141	48	45
S.E.	7	6	30	23	7	7	7	7	14	24	7	6

Table 14. Effect of intraarterial epinephrine infusion (61 ng/min)

Table 14. Effect of intraarterial epinephrine infusion (61 ng/min.) on gracilis muscle venous AMP. (Results, Figure 23)
Key: C = control; E = epinephrine

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	E_1	E_2	E_3	C	C	C	E_1	E_2	E_3	C
1	55	50	55	58	43	65	75	75	75	75	75	80
2	50	38	23	24	48	73	60	60	60	55	45	55
3	40	35	38	35	43	40	150	140	135	135	125	110
4	68	43	55	40	35	35	75	75	75	75	60	55
Mean	53	42	43	39	42	53	90	88	86	85	76	75
S.E.	6	3	8	7	3	9	20	18	17	17	17	13

Table 15. Effect of ADH on venous AMP. (Results, Figure 24)
Key: C = control; A = ADH

Dog #	Venous AMP, ng/ml						Perfusion Pressure, mm Hg					
	C	C	A_1	A_2	A_3	C	C	C	A_1	A_2	A_3	C
1	0	0	23	0	0	0	110	110	300	300	220	120
2	25	0	0	58	160	60	35	35	60	200	210	150
3	12	35	105	175	108	83	105	105	180	250	250	175
4	33	40	213	350	80	65	90	90	175	150	200	160
5	0	0	80	238	70	48	85	85	150	260	240	160
Mean	14	15	84	164	84	51	85	85	173	232	224	153
S.E.	7	9	37	63	26	14	13	13	38	26	9	9

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