METABOLISM OF ^{14}C-ATP ADDED TO DOG WHOLE BLOOD, PLASMA AND DURING PASSAGE THROUGH THE LUNGS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
ANN HAVILAND COLLINGSWORTH
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ABSTRACT

METABOLISM OF ^{14}C-ATP ADDED TO DOG WHOLE BLOOD, PLASMA AND DURING PASSAGE THROUGH THE LUNGS

By

Ann Haviland Collingsworth

Forrester and Lind (J. Physiol. 204: 347, 1969) and Chen et al. (Fed. Proc. 31: 379A, 1972) found ATP released into the venous effluent from exercising skeletal muscle and proposed that ATP was a chemical mediator for active hyperemia. Berne (Am. J. Physiol. 204: 317, 1963) reported that adenosine may be the mediator of coronary vasodilation induced by hypoxia. The metabolism of ATP in blood, plasma and upon passage through the vasculature of the lungs was therefore investigated to determine whether or not nanogram levels of ATP or its degradation products could recirculate. ^{14}C-ATP (575 ng/ml) was incubated in dog plasma or whole blood in vitro for accurately measured durations of time. ^{14}C-labeled nucleotide degradation products were separated and quantified by gradient elution ion exchange chromatography. The approximate halftimes for ^{14}C-ATP breakdown were 3 minutes in plasma and 1 1/2 minutes in whole blood. Breakdown products of ^{14}C-ATP in both whole blood and plasma were identified as ADP, AMP and nucleosides. After approximately 7 minutes incubation

in whole blood, extracellular ^{14}C-ATP degradation products were taken up by the formed elements of the blood and re-synthesized to intracellular ^{14}C-ATP. Heparin, barium and citrate had virtually no effect on ^{14}C-ATP breakdown in whole blood. Cooling the blood to 3°C significantly reduced ^{14}C-ATP breakdown. Approximately 17% of the added ^{14}C-ATP was broken down in 1 1/2 minutes at this low temperature as compared to 55% for controls incubated at 37°C. ^{14}C-ATP breakdown was inhibited in samples gassed with CO_2 as compared to higher pH control samples from the same dog. A mean decrease of 0.27 pH units inhibited ^{14}C-ATP breakdown 11% $\pm$ 2% (mean $\pm$ SE, N=16). Whole blood ^{14}C-ATP was most stable in the pH range of 7.20 to 7.42. Nucleosides and ADP were not affected in a consistent manner by whole blood pH alteration. ^{14}C-AMP was formed at decreasing rates as whole blood pH was lowered from 7.6 to 7.2. In vitro studies of ^{14}C-adenosine metabolism in whole blood revealed that ^{14}C-adenosine was rapidly taken up by the cells and synthesized into ^{14}C-AMP, ^{14}C-ADP and ^{14}C-ATP. Large quantities of ^{14}C-AMP were found in the plasma as well. Plasma ^{14}C-ATP levels were very low, if present at all. No ^{14}C-IMP was detected in plasma or cells. In vivo studies of ^{14}C-ATP degradation during a single passage through the vasculature of the lungs revealed that ^{14}C-ATP injected into the right atrium was almost completely broken down or taken up by the lungs. Of the ^{14}C activity injected

83% ± 3% (mean ± SE, N=4) was taken up by the lungs. Of the ^{14}C activity recovered in the blood after the passage of the ^{14}C-ATP injection through the lungs only 17% ± 11% (mean ± SE, N=4) was still ^{14}C-ATP. Other labeled ^{14}C-ATP degradation products in the blood leaving the lungs were nucleosides, 51% ± 14%; AMP (and/or IMP), 24% ± 6%; and ADP, 7% ± 3% (mean ± SE, N=4). The results indicate that ATP released from muscle tissue into the plasma could recirculate if its breakdown and uptake by the lungs could be inhibited.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	vii
INTRODUCTION	1
LITERATURE REVIEW	4
1. Pharmacological Effects of Adenine Nucleotides and Adenosine	4
2. Adenine Nucleotide Metabolism, General	37
3. Adenine Nucleotide Metabolism in Whole Blood and Plasma	37
4. Adenine Nucleotide Metabolism in Skeletal Muscle	56
5. Adenine Nucleotide Metabolism in the Heart	61
6. Metabolism of Extracellular Adenine Nucleotides by the Lungs	75
7. The Passage of Adenosine and Adenine Nucleotides Through the Cell Membrane	77
METHODS	84
1. Breakdown of ^{14}C-ATP in Plasma	84
2. Breakdown of ^{14}C-ATP in Whole Blood	85
3. Separation of Adenine Nucleotides by Ion Exchange Chromatography	85
4. ^{14}C-ATP Purity Check	92
5. ^{14}C-Adenosine Purity Check	92
6. Effect of pH on ^{14}C-ATP Breakdown in Whole Blood	95
7. IMP Separation	96
8. EDTA Studies	97
9. In Vivo Studies	98
(a) Plasma ^{14}C-ATP uptake and breakdown by the lungs	99

TABLE OF CONTENTS--Continued

	Page
(b) The effect of ATP injection and continuous infusion on systemic arterial blood pressure.	100
10. Reagents	101
RESULTS.	105
1. Breakdown of ^{14}C-ATP in Plasma	105
2. Breakdown of ^{14}C-ATP in Whole Blood.	110
3. Breakdown of ^{14}C-ATP in Blood with pH Controlled.	121
4. Studies of ^{14}C-ATP Breakdown in Paired Whole Blood Samples.	123
5. Effect of Barium, Citrate and Temperature on ^{14}C-ATP Breakdown in Whole Blood	123
6. Effect of Hemolysis on ^{14}C-ATP Breakdown . . .	128
7. Effect of pH on ^{14}C-ATP Breakdown.	128
8. Effect of EDTA on the Formed Elements of Blood	137
9. In Vivo Studies on ^{14}C-ATP Breakdown in the Pulmonary Circuit.	140
10. In Vivo Studies on the Effect of ATP on Systemic Arterial Pressure	143
DISCUSSION	147
SUMMARY AND CONCLUSIONS.	162
BIBLIOGRAPHY	164

LIST OF TABLES

TABLE		Page
1.	The Pharmacological Effects of the Adenine Compounds.	7
2.	^{14}C-ATP Breakdown at Selected Incubation Times in Whole Blood at 37°C	114
3.	Breakdown of ^{14}C-ATP in Whole Blood at 37°C in 1 1/2 Minutes with Uncontrolled pH	116
4.	^{14}C-ATP Breakdown in Whole Blood at 37°C in 1 1/2 Minutes at pH Approximating 7.40	122
5.	^{14}C-ATP Breakdown in Paired Whole Blood Samples from Individual Dogs	124
6.	Effect of Barium, Citrate and Temperature on ^{14}C-ATP Breakdown in Whole Blood in 1 1/2 Minutes.	126
7.	Effect of Hemolysis on ^{14}C-ATP Breakdown in Gassed Whole Blood in 1 1/2 Minutes.	129
8.	Effect of pH on ^{14}C-ATP Breakdown in Whole Blood in 1 1/2 Minutes	131
9.	The Effect of EDTA on Plasma ATP Levels. . . .	138

LIST OF FIGURES

FIGURE		Page
1.	Metabolism of the adenine compounds.	39
2.	Gradient elution ion exchange column chromatography apparatus	89
3.	Gradient elution ion exchange chromatography pattern for ATP, ADP, AMP, and adenosine	91
4.	^{14}C-ATP and ^{14}C-adenosine purity checks.	94
5.	^{14}C-ATP breakdown in isolated plasma incubated in vitro	107
6.	Breakdown of ^{14}C-ATP in isolated plasma incubated in vitro	109
7.	^{14}C-ATP breakdown in whole blood incubated in vitro.	112
8.	Distribution of ^{14}C activity in whole blood after 20 minutes of in vitro incubation with ^{14}C-ATP.	118
9.	Breakdown of ^{14}C-ATP in whole blood in 1 1/2 minutes at 37°C in vitro	120
10.	Effect of pH on ^{14}C-ATP breakdown in whole blood incubated in vitro at 37°C for 1 1/2 minutes . .	134
11.	Effect of pH on ^{14}C-ADP, ^{14}C-AMP, and ^{14}C-nucleoside formation in whole blood incubated in vitro with ^{14}C-ATP at 37°C for 1 1/2 minutes	136
12.	^{14}C-ATP breakdown during one passage through the vasculature of the lungs	142
13.	Effect of intravenous and intra-arterial ATP infusion on systemic arterial blood pressure . .	145

LIST OF ABBREVIATIONS

ATP = adenosine triphosphate

ADP = adenosine diphosphate

AMP = adenosine monophosphate

ITP = inosine triphosphate

IDP = inosine diphosphate

IMP = inosine monophosphate

μCi = microcurie

c/m = counts per minute

C.L.A.R. = Center for Laboratory Animal Resources

ng = nanogram

mg = milligram

kg = kilogram

μg = microgram

x G = times gravity

λ = .001 milliliter

SE = standard error (The standard errors in this thesis were calculated on per cent values. However, on comparison with standard errors obtained by using log values there was only a small difference observed between the two.)

2,3-DPGA = 2,3-diphosphoglyceric acid

GFR = glomerular filtration rate

g = gram

AV = auricular-ventricular

γ = microgram

INTRODUCTION

The studies presented in this thesis were done to investigate the metabolism of ATP in plasma, whole blood and during passage through the vasculature of the lungs. A more definitive understanding of adenine nucleotide metabolism in these tissues would greatly aid in determining the importance of ATP in the regulation of blood flow by allowing us to more accurately predict whether or not ATP, ADP, AMP and adenosine exist in arterial plasma. Also included in this thesis is a discussion of the possibility that extracellular ATP and other adenine compounds participate as mediators of physiological control. The evidence for the existence of adenine compounds in the extracellular fluid will be discussed, and the known pharmacological actions of these compounds will be reviewed. The most significant and most publicized pharmacological actions of the adenine compounds are related to the cardiovascular system and the local control of organ blood flow.

Conheim (1872) observed an increased blood flow in the frog's tongue following a short arrest of the circulation. The increased flow occurred in the absence of central influence. Gaskell (1877) noted changes in blood flow during and after skeletal muscle contraction. It was therefore suggested

that a locally produced metabolite was responsible for blood flow regulation in skeletal muscle (Gaskell, 1880, and Roy and Brown, 1879). A century later the cause of the active hyperemia in heart and skeletal muscle is still unknown. However, hypoxia, hypercapnia, hydrogen ion, potassium ion, Krebs cycle intermediates, osmolarity, prostaglandins, adenine and uridine compounds, and combinations of substances have all been suggested as participants in the increased blood flow. Adenine compounds (ATP, ADP, AMP and adenosine) have long been noted for having potent vasodilator properties as well as numerous other biological activities. Recent studies have provided evidence that certain adenine compounds may participate in the physiological regulation of blood flow in both heart and skeletal muscle. Jacob and Berne (1961) and Rubio, Berne and Katori (1969) found evidence that adenosine is the mediator of active hyperemia in the heart. Forrester and Lind (1969) found ATP released from the exercising human forearm. Chen et al. (1972) found ATP released during active hyperemia in skeletal muscle in the dog. Dobson et al. (1971), however, found adenosine and no ATP released from exercising skeletal muscle.

Chen et al. (1972) and Forrester and Lind (1969) also found evidence suggesting that ATP is a normal constituent of human and dog arterial plasma. Forrester (1972a), however, attributed his finding of arterial plasma ATP to platelet

damage and/or EDTA effects on the red cell. It is therefore still undetermined whether significant quantities of ATP are normally present in arterial plasma. Thus, the possibility of ATP (or other adenine compounds) in the arterial plasma entering the organs of the body and participating in the control of organ circulation and other physiological mechanisms must be considered.

Adenine compounds, if present in plasma, could produce significant vasodilation providing the organs of the body do not rapidly inactivate them. For example, the ATP found in the venous effluent of the exercising forearm could conceivably recirculate, resulting in vasodilation in many areas of the body. However, evidence indicating that ATP may be metabolized far more rapidly in blood passing through an organ than in blood alone has been published. Folkow (1949) and Gordon (1961) found indirect evidence suggesting that ATP could not survive passage through the lungs. Pfleger (1969) noted that 57 per cent of perfused adenosine was taken up by the lungs in 30 seconds. Liu and Feinberg (1971) found that 20 per cent of low levels of adenosine (0.3 uM) supplied to the heart in a perfusate was taken up by the myocardial cells. In the body, therefore, organ uptake of adenine compounds in the plasma could be considered quantitatively more important in their removal from the circulation than their breakdown in the blood itself.

LITERATURE REVIEW

1. Pharmacological Effects of Adenine Nucleotides and Adenosine

The role of ATP as the driving force of many biochemical processes has been recognized and investigated since its proposal by Lipmann (1941). Also of historical interest was the recognition prior to 1930 of the importance of the adenine compounds in the glycolytic process and the relationship between glycolysis and muscle contraction (Meyerhoff, 1920); see Bodansky (1927) for a brief summary of Meyerhoff's studies.

Concomitant with this development of our understanding of the biochemical importance of the adenine compounds, it is noteworthy that as early as 1929 the adenine compounds were recognized to have numerous pharmacological effects on the mammalian body by Drury and Szent-Gyorgyi (1929) working at Cambridge, England. Drury and Szent-Gyorgyi isolated a substance from acid extracts of heart muscle and other tissues, which produced heart block and bradycardia upon intravenous injection. This biologically active substance was identified by these investigators to be the nucleotide adenylic acid (AMP). Other pharmacological effects of intravenous AMP injection which were noted by Drury and Szent-Gyorgyi included: a decrease in arterial blood pressure due both to arteriolar

dilation and bradycardia; arrest of intestinal peristalsis; coronary vasodilation; less forceful auricular contraction; arrest of experimentally produced auricular fibrillation; shortened auricular absolute refractory period; and decreased renal blood flow and urine formation. Adenosine, ADP, and ATP were found by these investigators to have pharmacological properties similar to those of adenylic acid; however, the removal of the ribose of adenosine to form adenine, the removal of the amine to form inosine, and the transfer of the amine to form guanosine all removed the biological activities of adenosine described above.

Many of the initial findings reported by Drury and Szent-Gyorgyi were repeated and extended by Bennet and Drury (1931), who made the following additional observations with AMP and adenosine obtained from muscle and yeast extracts: "heart block" in the guinea-pig; dilation of .03-.05 mm diameter blood vessels in the intact and isolated perfused rabbit ear; vasoconstriction in the isolated, perfused rabbit lung; both vasoconstriction and dilation in the isolated, perfused rabbit kidney*; bronchiolar dilation; relaxation of the guinea-pig gall bladder; contraction of the isolated virgin guinea-pig uterus; decreased guinea-pig rectal temperature and localized accumulation of leucocytes in the subcutaneous

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*This variability may be explained by ATP and ADP contaminates in their relatively crude preparations of adenosine and AMP.

area where nucleoside or nucleotide were applied. The relation of this last observation to the inflammatory process was discussed by these investigators.

After the above publications described the "heart block" and other biological effects caused by adenosine and adenine nucleotides, the obvious importance of these phenomena led to the further investigation of the pharmacology of the adenine compounds by several laboratories during the early 1930's. These studies were extensively reviewed by Drury in 1936 and Table 1 summarizes the observations reviewed by this author as well as some of the other recent findings related to the pharmacology of the adenine compounds. Some of the highlights of these studies listed in Table 1 which relate to this thesis will now be discussed.

J. H. Gillespie published an interesting article in 1933 dealing with the "biological significance of the linkages in adenosine triphosphoric acid." In addition to presenting a worthwhile review of the pharmacology of the adenine compounds known at that time (including references to several papers unavailable to us), a number of unique experimental observations were recorded. In the cat Gillespie noted a biphasic response of arterial pressure to ATP intravenous injection, i.e., first an increase followed immediately by a decrease. When a second bolus of ATP was given intravenously, only a rise in blood pressure was observed. ATP, unlike

Table 1. The Pharmacological Effects of the Adenine Compounds

		Skeletal Muscle	
increased blood flow (forearm)	ATP	Duff et al.	(1954)
decreased small vessel resistance (forelimb)	AMP, ADP ATP	Frohlich	(1963)
generalized muscular contraction	ATP	Emmelin & Feldberg	(1948)
vasodilation (hindlimb)	AMP, ATP	Kontos et al.	(1968)
increased capillary filtration coefficient	ATP	Kjellmer & Odelram	(1965)
dilated capacitance vessels	ATP	Kjellmer & Odelram	(1965)
		Lung	
vasoconstriction	AMP, adenosine	Bennet & Drury	(1931)
vasodilation (transient)	AMP, ADP, ATP (low dose)	Gaddum & Holtz	(1933)
vasoconstriction (lasting)	AMP, ADP, ATP (High dose)	Gaddum & Holtz	(1933)
vasoconstriction	ATP	Emmelin & Feldberg	(1948)
decreased pulmonary artery pressure	ADP	Brashear & Ross	(1969)
increased pulmonary artery pressure	ADP	Brashear et al.	(1970)
		Nerves	
increased splanchnic nerve activity	ATP	Dontas	(1955)
increased chemoreceptor activity	ATP	Dontas	(1955)
initiates scratch & other reflexes	ATP	Emmelin & Feldberg	(1948)
increased chemoreceptor activity	ATP	Jarisch et al.	(1952)
increased baroceptor activity	ATP	Jarisch et al.	(1952)

continued

Table 1--Continued

		Heart	
heart block	AMP adenosine	Drury & Szent-Gyorgyi Bennet & Drury	(1929) (1931)
bradycardia	AMP adenosine	Bennet & Drury	(1931)
coronary vasodilation	AMP adenosine	Bennet & Drury	(1931)
arrest of auricular fibrillation	AMP adenosine	Bennet & Drury	(1931)
shortened auricular absolute refractory period	AMP adenosine	Bennet & Drury	(1931)
coronary vasodilation	ATP	Wolf & Berne	(1956)
bradycardia (not via vagus)	ATP	Emmelin & Feldberg Bielschowsky et al.	(1948) (1944)
impaired AV conduction	ATP adenosine	Urthaler & James	(1972)
bradycardia	ADP	Brashear & Ross	(1969)
increased O_2 consumption	adenosine	Wedd & Fenn	(1933)
decreased amplitude of contraction	adenosine AMP	Wedd & Fenn	(1933)
increased beat strength (frog)	ATP AMP	Parnas & Ostern	(1932)
occasional increased beat strength (rabbit)	ATP AMP	Drury	(1932)
bradycardia (frog, perfused)	ATP, AMP adenosine	Ostern & Parnas	(1932)
coronary dilation	adenosine, AMP adenine	Wedd	(1931)
		Kidney & Bladder	
decreased renal blood flow	AMP adenosine	Drury & Szent-Gyorgyi	(1929)
decreased urine flow	AMP adenosine	Drury & Szent-Gyorgyi	(1929)

continued

Table 1--Continued

vasoconstriction	AMP adenosine	Bennet & Drury	(1931)
vasodilation	AMP adenosine	Bennet & Drury	(1931)
vasoconstriction	adenosine	Buyniski & Rapela	(1969)
micturation	ATP	Emmelin & Feldberg	(1948)
initial decreased RBF	AMP adenosine	Tagawa & Vander	(1970)
decreased GRF	AMP adenosine	Tagawa & Vander	(1970)
sodium excretion decreased	AMP adenosine	Tagawa & Vander	(1970)
renal venous renin decreased	AMP adenosine	Tagawa & Vander	(1970)
increased RBF	ATP	Tagawa & Vander	(1970)
decreased GRF	ATP	Tagawa & Vander	(1970)
decreased Na excretion	ATP	Tagawa & Vander	(1970)
decreased renal venous renin	ATP	Tagawa & Vander	(1970)
vasoconstriction	adenosine	Scott et al.	(1965)
vasodilation	ATP	Scott et al.	(1965)
vasoconstriction and vasodilation	AMP ADP	Scott et al.	(1965)
vasoconstriction	adenosine	Marcou	(1932)
bladder contraction	ATP	Burnstock et al.	(1972)
	Uterus		
contraction	AMP adenosine	Bennet & Drury	(1931)
contraction	ATP	Gillespie	(1933)

continued

Table 1--Continued

		Gall Bladder	
relaxation	AMP adenosine	Bennet & Drury	(1931)
		Body Temperature	
decreased (rectal)	AMP adenosine	Bennet & Drury	(1931)
increase (forearm skin)	ATP	Stoner & Green	(1945)
increase	adenosine	Richards	(1934)
		Stomach	
decreased rythmic motility	adenine, adenosine AMP, ADP, ATP	Rehm et al.	(1970)
vomiting	ATP	Emmelin & Feldberg	(1948)
		Intestine	
decreased peristalsis	AMP adenosine	Drury & Szent-Gyorgyi Gillespie	(1929) (1933)
increased tone	ATP	Gillespie	(1933)
defecation	ATP	Emmelin & Feldberg	(1948)
decreased activity	adenosine	Werle & Schievelbein	(1964)
decreased vascular resistance	adenosine ATP	Chou	(1966)
decreased compliance	adenosine	Chou	(1966)
increased ileal motility (high dose)	ATP adenosine	Chou	(1966)
		Platelets	
decreased clumping	ATP, AMP adenosine	Born & Cross	(1963)
increased clumping	ADP	Born & Cross	(1963)

continued

Table 1--Continued

		Respiratory Center	
transient cessation followed by increased ventilation	ATP	Emmelin & Feldberg	(1948)
no effect on ventilation	ATP (low dose)	Folkow	(1949)
increased ventilation	ADP	Brashear & Ross	(1969)
decreased tidal volume	ADP	Brashear & Ross	(1969)
		Systemic Vasculature	
decreased arterial pressure	AMP adenosine	Drury & Szent-Gyorgyi Bennet & Drury	(1929) (1931)
vasodilation	AMP adenosine	Drury & Szent-Gyorgyi	(1929)
biphasic increase & decrease in arterial pressure	ATP	Gillespie	(1933)
decreased arterial pressure (cat)	ATP	Emmelin & Feldberg	(1948)
decreased arterial pressure	ATP	Folkow	(1949)
decreased arterial pressure	AMP	Kalckar & Lowry	(1947)
decreased arterial pressure (rat)	AMP, adenosine ADP, ATP	Gordon & Hesse	(1961)
decreased arterial pressure (cat)	AMP, ADP, ATP	Bielschowsky et al.	(1944)
		Brain	
very slight vasodilation	adenosine	Buyniski & Rapela	(1969)
increased electrical activity	ATP	Benzi et al.	(1969)
		Skin	
dilation (rabbit ear)	AMP adenosine	Bennet & Drury	(1931)
accumulation of leucocytes	AMP adenosine	Bennet & Drury	(1931)

continued

Table 1--Continued

	Isolated Arterial Strips		
relaxation (facial artery)	AMP, ADP ATP, 3,5-AMP	Gebert et al.	(1969)
relaxation (renal artery)	adenosine, AMP, ATP	Collingsworth & Selleck	(Unpub. Observ.)
relaxation (femoral artery)	adenosine, ATP	Collingsworth & Selleck	(Unpub. Observ.)
	Spleen		
decreased volume (isolated perfused)	adenosine	Marcou	(1932)

adenosine and AMP, was observed to frequently increase the 'tone' of the isolated small intestine. One intestinal preparation described by Gillespie "had no sign of life until the ATP was added". In studies performed on the isolated virgin guinea-pig uterus, ATP, AMP and adenosine all caused increased contraction, a phenomenon reported earlier by Bennet and Drury (1931). Isosine, IMP and ITP were also studied by Gillespie and his experiments with these latter compounds demonstrated qualitatively similar responses to those with the adenine compounds; however, a ten-fold greater dose was required in the case of the inosine compounds to give observable effects. Purity checks and anesthetics were not reported in this paper.

Gaddum and Holtz (1932-1933) found that small doses of adenine compounds (5 µgram) in cats and dogs produced transient vasodilation in the isolated, blood perfused lung; while larger doses (250 µgram) produced lasting pulmonary vasoconstriction. The effect was apparently on the pulmonary arterioles, but some qualitatively similar changes in pulmonary venous tone were also observed. Adenylpyrophosphate (ADP and ATP) effects were much more pronounced than those of adenosine or AMP.

Emmelin and Feldberg (1948) studied systemic effects of ATP in decerbrated and chloralosed cats. They noted a steep fall in arterial blood pressure upon intravenous injection of

.2 to .4 mg of ATP. This was attributed to pulmonary constriction, bradycardia, and systemic vasodilation. It was also observed that much smaller doses of ATP could cause decreased systemic arterial blood pressure when injected into the left auricle rather than intravenously. A direct effect of ATP on the respiratory center was also suggested, based upon respiratory changes after ATP injection into the carotid and vertebral arteries. These changes consisted of initial cessation of respiration or of shallow frequent respiration followed by hyperventilation. There was also evidence for indirect effects on the respiratory center mediated via the vagi. The possibility that ATP may stimulate the chemoreceptors was discussed but not experimentally studied.

Folkow (1949) observed that ATP was a powerful vasodilator in cats and dogs. The threshold dose for the effect was 0.05 to 0.1 γ when injected intraarterially, a potency about one-fifth to one-fifteenth that of acetylcholine. The small doses used by Folkow produced no significant effects on the heart or on respiration. Atropine, neoantergan and vessel denervation did not block the dilator effect of ATP, implying but not proving a direct effect on the vessels. Intravenous injection of low doses of ATP produced no decrease in systemic arterial pressure, indicating inactivation of ATP by the lungs. Folkow suggested a possible role of ATP

in peripheral vascular regulation, a theory discussed earlier by Zipf (1931) and Rigler (1932). Although circumstantial supportive evidence for adenyl compounds being the mediators of reactive and active hyperemia was presented by these early investigators, Drury (1936) states that there is no proof. These early workers proposed the interesting theory that during muscle contraction ATP is degraded to AMP, and AMP then passes out of the cells and causes hyperemia. The finding of Dale (1933) that AMP is less active than ATP did not support the theory, but Gillespie's observation that AMP was more active than ATP did. More recently support for the proposal that ATP is the mediator comes from Duff et al. (1954), who found a three-fold increase in muscle blood flow after infusion of 16 µg ATP/min. into the human forearm. The vasodilator properties of ATP, acetylcholine, and histamine were compared. Mg-ATP caused as large an increase in forearm blood flow as did acetylcholine or histamine, but without the uncomfortable side effects associated with the latter two drugs. Mg-ATP was a more powerful vasodilator than Na-ATP. This was attributed to the potentiated vasodilator action of ATP when combined chemically with magnesium. Mg-ATP did not appear to be inactivated by the circulation as rapidly as acetylcholine but was inactivated more rapidly than histamine. Interestingly, Mg^{++} is a dilator even when injected unbound to ATP. Wolf and Berne (1956) achieved

maximal vasodilation with an ATP infusion of .2 to .3 uM/min into the coronary arteries. It is thus obviously possible that small quantities of ATP released from endogenous sources could participate in regulation of blood flow.

Gordon (1961) and Frohlich (1963) localized the vasodilator effect of the adenine nucleotides to small vessels, particularly the arterioles. Kjellmer and Odelram (1965) found changes in both arterial and venous resistance associated with ATP-induced dilation. A slow movement of intravascular fluid into the tissue spaces was also found.

Scott et al. (1965) provided evidence that ATP may be involved in regulating blood flow through kidney, hindlimb, and heart. Using a bioassay organ technique, they found that bioassay organ resistance changed in the same direction when the assay organ was the forelimb and in the opposite direction when the assay organ was the kidney. This finding suggested that active hyperemia, reactive hyperemia, and autoregulation of blood flow may result from a change in the chemical environment of the vessels--an extension of the theory proposed by Gaskell (1880).

Large doses of ATP affect the heart. Green and Stoner (1950) found that ATP initially reduced cardiac contractility, followed by a period of augmented contraction. Urthaler and James (1972) found a negative dromotropic action of ATP on the heart. This effect was directly related to the number of

attached phosphate groups. A purine nucleus and 6-amino group aided this effect. Urthaler and James (1972) found further effects of adenine compounds on the heart. All adenine nucleotides impaired AV conduction. ATP and adenosine produced negative chronotropic action. Transient ectopic beats of AV junctional origin were observed at the onset of AV block. Phosphate bonds were not necessary for the chronotropic effect of ATP. It was suggested that ATP and adenosine exerted their effect by modification of the myocardial cell membranes. The negative chronotropic effect of ATP occurred at much lower concentrations than the negative dromotropic effect. ATP injected in a concentration of 1 to 10 mg/ml produced an immediate heart block of 5 to 30 seconds duration. It was suggested that since ATP is concentrated within myocardial cells, their destruction during an infarct could release sufficient ATP to cause further heart block.

Although the pharmacological effects of the adenine compounds have not been studied extensively in the kidney, several papers of interest have appeared. Decreased renal blood flow and urine flow with intravenous AMP were observed by Drury and Szent-Gyorgyi (1929). Similar findings (adenosine and AMP increase and ATP decreases renal resistance) have been reported by Scott et al. (1965), Thurau (1964), Hashimoto and Kumakura (1965), Nechay (1966) and Harvey (1964).

Gordon (1962) found AMP in venous plasma from the rabbit kidney after brief arterial occlusion, implicating AMP in the autoregulation of renal blood flow. However, in these studies of Gordon rate of renal blood flow influenced the amount of AMP, with AMP present at high rates of flow only. This could indicate rapid breakdown of extracellular AMP in the kidney. Recently a more complete study of the effects of the adenine compounds on renal blood flow has been published by Tagawa and Vander (1970). These investigators also found initially a decrease in renal blood flow with adenosine and AMP; however, steady infusion of these compounds into the renal artery did not change or caused a slight increase in renal blood flow. ATP infusion increased renal blood flow with no initial decrease. Adenosine and AMP infusion lowered GFR and sodium excretion and renal venous renin activity. ATP infusion also decreased GFR, sodium excretion and renal venous renin. It seems, therefore, that ATP dilates the efferent arterioles. On the basis of these findings, Tagawa and Vander suggest that adenosine and/or AMP may be the normal mediators of both autoregulation and renin secretion--thus overlapping but also conflicting their proposal with that of Gordon's (1962). This hypothesis suggests that AMP or adenosine released during increased renal perfusion pressure could cause afferent arteriolar vasoconstriction in an effort to maintain GFR and reduce renal blood flow

simultaneously. Also suggested was the possibility that when renal perfusion pressure is increased; more sodium is filtered; more sodium is reabsorbed; and thus more ATP breaks down to AMP. AMP and adenosine thus formed then pass out of the tubular cells, cause autoregulatory vasoconstriction and also cause decreased renin. It is difficult to understand if renal venous plasma AMP increases with brief renal arterial occlusion, as reported by Gordon, how renin release increases in this situation because Tagawa and Vander report that AMP inhibits renin production by the kidney. Further evidence implicating adenosine and AMP in renal blood flow autoregulation is discussed by Ono et al. (1966). These investigators observed that dipyridamole decreases renal blood flow.

Several investigators have studied the action of adenine compounds in the lung. Brashear and Ross (1969) found that injection of 12 mg/kg of ADP into the pulmonary artery caused a sustained fall in pulmonary artery pressure lasting for 30 minutes and systemic hypotension lasting 5 minutes. ADP disappeared much more rapidly in vivo (one minute) than in vitro (20 minutes), although it did survive passage through the lungs, since arterial ADP levels were significantly higher than controls immediately after ADP injection into the pulmonary artery. Heart rate decreased for five minutes after injection. However, Brashear et al. (1970) found increased pulmonary artery pressure, cardiac output, central blood

volume, stroke volume and heart rate upon injecting .4 mg/min/kg into the femoral vein. Decreases in aortic pressure, systemic resistance, and platelets also occurred. The increased pulmonary artery pressure resulted from increased cardiac output rather than increased resistance, as no change in pulmonary vascular resistance was recorded and pulmonary artery pressure promptly returned to control levels although circulating platelet levels remained depressed. Therefore, the decrease in platelets did not seem to be related to the rise in pulmonary artery pressure. Gordon (1961) found that ADP was a more potent vasodilator than ATP, AMP, or adenosine upon intravenous injection. With intra-arterial injection, ATP and ADP were equally potent, indicating that ADP may pass through the lungs whereas ATP may not.

During the 1930's the finding that adenine compounds lowered arterial blood pressure led to the proposal that these compounds may also be responsible for traumatic shock. This theory was supported by the large quantities of adenyl compounds in tissue--injury of the tissue would supposedly release these compounds into the blood and thus cause decreased blood pressure. Bennet and Drury (1931) observed release of adenosine-like substances from burned, perfused rabbit heart using a guinea pig heart as a bioassay. Similar findings were made by Zipf (1932), who in 1931 published the above suggestion, as did Konig (1930). H. N. Green (1943),

Bielschowsky and Green (1943) and Bielschowsky and Green (1944) discuss and provide experimental support for the theory that ATP is one of the chemically labile shock-producing factors from striated muscle--these studies perhaps were stimulated by war injuries. With the development of sophisticated assays for the adenine nucleotides, Kalckar and Lowry (1947) attempted to chemically assay these materials in plasma during 'traumatic' shock. Anesthetized dogs and rabbits were submitted to leg injuries (500-800 blows with a mallet). Four to five minutes after such traumatization blood pressure decreased, and the animals became weak and drowsy. In several cases "slight but distinct" increases in the concentration of adenine compounds were observed in venous plasma from the traumatized extremity. No increase in systemic arterial plasma adenine compounds was observed even though blood pressure was greatly decreased. Furthermore, injection of adenosine deaminase had no effect on blood pressure in these animals. Although Kalckar and Lowry state that it is unlikely that adenylic acid compounds play a primary role in traumatic shock, their findings did not rule out these substances as "secondary factors". Staples et al. (1969) have noted decreased ATP in skeletal muscle, liver and kidney with both hemorrhagic and endotoxin shock.

This review of the pharmacological actions of the adenine compounds has not included cyclic 3,5-AMP and adenosine

tetraphosphate. A vast amount of information concerning the biological actions of cyclic 3,5-AMP has recently developed, research in this area being stimulated perhaps by the finding of Sutherland and Rall (1960) that cyclic 3,5-AMP is an intracellular mediator for the glycogenolytic action of epinephrine. A short review dealing with the biological effects of cyclic AMP has been published by Butcher (1968). No information concerning the pharmacology of adenosine tetraphosphate was found, although it is present in tissue and is available commercially.

Due to the vasodilator property of the adenine compounds, it has been suggested that they may be involved in peripheral vascular regulation. Stainsby (1973) presents an excellent review of the control of peripheral blood flow. Although oxygen, carbon dioxide, potassium ion, Krebs cycle intermediates, hydrogen ion and osmolarity have also been suggested as possible regulators of blood flow, Scott et al. (1965) found evidence that physiological changes in oxygen, hydrogen ion, sodium and potassium were not adequate to produce the increased blood flow which accompanies exercise. Frolich (1965) found a significant vasodilator effect of Krebs cycle intermediates on small vessels. However, the degree of change of these intermediates during physiological alteration of blood flow is not known.

Jacob and Berne (1961) proposed that adenosine may be the mediator of coronary vasodilation in the hypoxic heart. Also, Berne (1963) in his early studies suggested that AMP may be involved in the regulation of skeletal muscle blood flow since no adenosine was found in either normal or anoxic muscle. However, adenosine has recently been reported to be released during ischemic contraction in skeletal muscle (Dobson et al., 1971). IMP was found in normal muscle and increased during anoxia, with an associated increase in inosine and hypoxanthine levels. A sharp drop in ADP also occurred in exercising skeletal muscle, possibly due to IDP formation (Imai et al., 1964) as well as degradation to AMP. Kontos et al. (1968), however, presented evidence but not proof that AMP and ATP were not responsible for the vasodilation which occurred in skeletal muscle during short periods of ischemia. They found that dipyridamole, which potentiated the vasodilator effect of AMP and ATP, did not augment the vasodilator response to short periods of ischemia.

Adenosine, like AMP, has numerous effects on the mammalian cardiovascular system, as summarized in Table 1. In addition to causing vasodilation throughout most of the body, adenosine was found to vasoconstrict the kidney (Scott et al., 1965), the lung (Bennet and Drury, 1931) and possibly the spleen (Marcou, 1932). Bradycardia and a negative inotropic effect on the heart have also been noted with adenosine.

Wolf and Berne (1956) found that adenosine and AMP were equally potent vasodilators, but were only one-fourth as effective as ADP and ATP in increasing coronary blood flow. One wonders if the more potent dilators are not the ones most active normally in the body.

Several drugs have been found to potentiate the vasodilatory effect of adenosine. Persantin (dipyridamole) and lidoflazine are two such drugs. Bunag et al. (1964) found that the drug Persantin prevented adenosine deamination by erythrocytes, presumably due to reduced permeability of the erythrocyte membrane to adenosine. Afonso and O'Brien (1971) found that dipyridamole and lidoflazine delayed the disappearance of adenosine in blood. The main effect of these drugs, however, was a decrease in tissue permeability to adenosine, especially in the lungs. Since these drugs cause vasodilation, it is tempting to speculate that adenosine compounds are normally present in the extracellular fluid.

Forrester (1966) provided the first direct evidence that ATP is released from active frog skeletal muscle in vitro. Further studies by Boyd and Forrester (1968) supported the concept that the ATP did not come from muscle cell damage, as potassium ion level in the plasma did not increase. They suggested that ATP release may proceed via the transverse tubular system of the sarcoplasmic reticulum in skeletal

muscle. Forrester and Lind (1969), using firefly extract analysis for ATP, identified ATP in human plasma, both in resting and exercising subjects. Venous ATP levels of exercising subjects rose consistently above resting values, indicating addition of ATP to blood passing through the muscle bed. Forrester (1972a) 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 exercising skeletal muscle, 0.033 to 1.0 nmole ATP/ml of plasma was observed. After taking into account the amount of ATP lost by degradation, it was concluded that ATP could be the mediator of the active hyperemia seen in the human forearm.

Chen et al. (1972) also found ATP in the venous effluent during active hyperemia in skeletal muscle. Control ATP levels were 206 ng/ml plasma in the femoral artery and 165 ng/ml plasma 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 approximately 188 ng/ml plasma. Increased flow correlated with increased ATP levels.

ADP released from platelets and/or tissue cells at a site of injury or hemorrhage initiate a series of events which participate in hemostasis (Ganong, 1969). In the presence of ADP, platelets clump together at the hemorrhage

site forming a temporary plug. Also occurring simultaneously with the release of ADP from the platelets is the release of serotonin, which theoretically causes a potent vasoconstriction in the area of the hemorrhage. In addition to ADP, other adenine nucleotides have been observed to affect the clumping of platelets--some of these observations relating the adenine compounds to platelet function are described below.

Zuker and Borrelli (1960) demonstrated that ADP added to plasma resulted in platelet swelling. Born and Cross (1963) found that ADP added to plasma caused rapid platelet aggregation. The platelets dispersed with time, and it was proposed that ADP breakdown was the cause. It was suggested that ADP associated with "aggregating sites" on the platelet surface. AMP and adenosine inhibited aggregation, adenosine ten times as effectively as AMP. ATP was slightly inhibitory. The inhibition could have resulted from competition for the aggregation sites. The ADP, AMP, adenosine and ATP exerted their effects in very low concentrations. The reactions were highly specific. EDTA inhibited the aggregation effect. Calcium addition sometimes caused aggregation.

Salzman et al. (1966) further investigated the effect of ADP on platelets. They proposed that ADP caused platelet aggregation by product inhibition of ATP breakdown by ecto-ATPases in the platelet membrane. Blocking this energy-supplying reaction would result in the platelets losing their unique shape and becoming spherical. This could expose

adhesive sites on the platelet membrane. ADP breakdown would stop product inhibition of the ATP breakdown and allow the platelet to assume its normal shape. Evidence for this theory was found in the facts that neither ADP breakdown nor ADP binding to the platelets was required for aggregation. AMP was inhibitory only after dephosphorylation to adenosine. The adenosine inhibition may be due to an adenosine-carrier complex crossing the platelet membrane. ATP also inhibited platelet aggregation.

Spaet and Lejneiks (1966) proposed that the breakdown of ADP to AMP was the cause of ADP-induced aggregation. EDTA, AMP, and removal of plasma all prevented platelet clumping, thus supporting this theory.

Hellem and Owren (1964) proposed a complex bridging between platelets occurred during aggregation. Calcium ions, ADP and von Willebrand's factor presumably composed the bridge. Experimental evidence did not support this theory, however (Spaet, 1965).

Haslam (1964) showed that the aggregating effect of thrombin on platelets was mediated by ADP release from the platelets. Ireland (1967) further studied thrombin effects on platelet adenine nucleotides. The results showed that two nucleotide pools probably exist in platelets. Thrombin treatment caused release of largely non-radioactive nucleotide pools. The two pools contained approximately the same amounts

of ATP and ADP. The ATP:ADP ratio in the thrombin-released pool was 0.7-0.8.

Karpatkin and Langer (1968) found that both thrombin and epinephrine caused release of sufficient ADP to account for platelet agglutination. They also found a high rate of ATP utilization in platelets.

Holmsen (1967) found further evidence for the existence of two nucleotide pools in platelets. One pool participated in metabolism and was not lost during clumping. P^{32} taken up by the platelets was found in this nucleotide pool, and a large proportion of these nucleotides was protein bound. The second pool did not participate in metabolism but was released upon external stimulation. Approximately 2/3 of platelet nucleotides belonged to the second pool. ADP released by collagen did not come from platelet ATP but presumably from the large platelet ADP stores.

Born and Cross (1963) found that repeated addition of small amounts of ADP to plasma diminished the aggregating effect of ADP. Rosenberg and Holmsen (1968) also found a loss of platelet aggregability upon repeated addition of ADP. This effect was termed the refractory state of the platelets. The refractory condition remained long after ADP breakdown and was due to ADP rather than its breakdown products.

When viewed together, the pharmacological effects of the adenine compounds seem to indicate that in many preparations these compounds depress muscle contraction. In the

gall bladder relaxation was observed by Bennet and Drury (1931); in the stomach decreased rhythmic motility was noted by Rehm et al. (1970); in many tissues vasodilation was reported; in the intestine decreased activity was noted by Werle and Schievelbein (1964); and in isolated arterial strips Gebert et al. (1969) observed relaxation. These findings suggest that the adenine compounds may have a basic action on smooth muscle in general and perhaps are similar in biological function to acetylcholine. The release of ATP from nerves has been reported by Holton (1959), from the adrenal medulla with adrenal nerve stimulation by Douglas (1966), from frog nerve muscle preparations with electrical stimulation by Abood et al. (1962) and from non-adrenergic inhibitory nerves in the gut by Burnstock et al. (1970). Therefore, nerves may serve as a direct source of ATP or at least an indirect source by mediating its release from other cells.

For many years there have been hints that nerves other than the classical adrenergic and cholinergic types are present in the autonomic nervous system. However, their effects were frequently attributed to the presence of sympathetic nerves running in vagal trunks or to special receptor sites rather than to a third type of nerve. It has recently been demonstrated that a third type of nerve does exist in the autonomic nervous system (Robinson et al., 1971). These

nerves were distinguished by a different type of vesicle than that found in adrenergic, cholinergic or sensory nerves. The vesicles were large, granular and opaque. Application of 6-hydroxydopamine, which resulted in degeneration of adrenergic nerve fibers, did not affect the non-adrenergic nerve profile; the vesicles were still present and the inhibitory response to stimulation remained. Reserpine, which depletes catecholamine content, also had no effect on these nerves. It was therefore concluded that this type of nerve was neither cholinergic nor adrenergic but represented a third and distinct type of nerve. Burnstock (1972), in an excellent review of the subject, terms this class of nerves "purinergic", since there is evidence that ATP is their neurotransmitter (Burnstock et al., 1970).

Extensive evidence for the existence of purinergic nerves comes from studies on the gastro-intestinal tract. Atropine did not affect inhibitory potentials in the guinea-pig taenia coli (Burnstock et al., 1963). Campbell (1966) found that low frequency vagal stimulation of the atropinized guinea-pig stomach was much faster and more effective in producing relaxation than perivascular (sympathetic) nerve stimulation. Bretylium abolished the response to perivascular stimulation but did not affect the response to vagal stimulation. Beani et al. (1971) also studied purinergic nerve effects in the guinea-pig stomach. Atropinization reversed vagal stimulatory effects from excitatory to inhibitory. Sympathetic

blocking agents (guanethedine, bretylium, reserpine) did not abolish vagal inhibition. The results of these studies suggest, therefore, that vagal purinergic nerves may be present in the stomach; their function here is probably related to control of gastric motility.

Studies on the intestine have indicated that purinergic nerves are located in this tissue also. Ambache (1951) noted that addition of botulinum toxin, which paralyzes cholinergic nerves, produced evidence which revealed a group of inhibitory nerves in the enteric plexuses of mice and rabbits. The response to nicotine in a normal intestine was excitatory, whereas in a botulinum-poisoned preparation nicotine produced inhibition of intestinal motility. However, these inhibitory nerves were considered to be sympathetic in origin by Ambache. Burnstock et al. (1964) found electrophysiological evidence for two types of inhibitory nerves in the guinea-pig taenia coli. Intramural inhibitory nerves responded to lower transmural stimulatory frequencies than sympathetic nerves and were not affected by adrenergic blocking agents, as were the sympathetic nerves. Everett (1968) reported evidence for the presence of purinergic nerves in the ileum and rectal caecum of the chick. Burnstock et al. (1966) identified two types of inhibitory nerves in the smooth muscle of the guinea-pig taenia coli: perivascular and intramural inhibitory nerves. The cell bodies of the intramural inhibitory nerves

were localized in Auerbach's plexus. Both circular and longitudinal muscle were innervated by the intramural nerves (Furness, 1969). Bulbring and Tomita (1967) noted that the intrinsic (intramural) nerve fibers were quite short (a few mm), based on electrophysiological studies. Addition of tetrodotoxin, which blocks nerve but not smooth muscle function, abolished inhibitory potentials in the smooth muscle, thereby ruling out the possibility that muscle stimulation by itself produced the inhibitory potential. The inhibitory potentials were not blocked by guanethedine (Furness, 1969) and therefore were not of sympathetic origin.

Electrophysiological studies have revealed some characteristics of the inhibitory potentials produced by 'purinergic' nerves. Bennett et al. (1966) found that stimulation of short duration (200 μsec) excited intramural inhibitory nerves of the guinea-pig taenia coli but not the muscle. Hyperpolarization of the smooth muscle occurred in response to single stimuli applied to the intramural nerves but not to single stimuli applied to the perivascular nerves. The latency of response was longer in intramural nerves. Beani et al. (1971) found a rebound excitation following an inhibitory potential; this consisted of depolarization beyond the normal level and therefore a state of increased contraction. Simultaneous stimulation of excitatory and inhibitory neurones in the stomach results in contraction (Beani et al., 1971) whereas it causes relaxation in the colon (Furness, 1969).

Purinergic nerves have also been implicated in other organs. Robinson et al. (1971) found evidence to suggest the existence of such nerves in the lung. Burnstock et al. (1972) found the urinary bladder contained atropine-resistant nerves which were excitatory. Nerves with purinergic characteristics have also been located in the avian gizzard (Bennett, 1969). The presence of purinergic nerves in the cardiovascular system is as yet only speculative. The function of these nerves is uncertain; however, their participation in the regulation of organ motility seems highly probable.

Evidence has accumulated which indicates that ATP is the neurotransmitter released by purinergic nerves. Indirect evidence comes from studies of the effect of ATP on specific organs. Where the stimulation of the proposed purinergic nerves results in relaxation, infusion of ATP also results in relaxation. This was demonstrated in the gut by Burnstock et al. (1970). In organs where stimulation of the proposed purinergic nerves results in contraction, ATP infusion also results in contraction, as shown by Burnstock et al. (1972) in the urinary bladder. The bladder response to ATP was also similar to the nerve-mediated response; both were characterized by rapid contraction and rapid return to the original condition. Quinidine blocked both response to stimulation of the proposed purinergic nerves and to ATP. ATP was the most potent adenine compound. Satchell et al. (1969)

identified adenosine in the perfusate of stomachs in which inhibitory nerves had been stimulated, suggesting that the adenosine may have been coming from the nerves; however, it could have been coming from the other areas of the stomach as well.

More direct evidence comes from studies of Burnstock et al. (1970). Stimulation of inhibitory nerves was accompanied by the presence of adenosine and inosine in stomach perfusates. Satchell and Burnstock (1971) found that ATP breakdown by the stomach could account for observed levels of adenosine and inosine. ATP, ADP and AMP were found in the medium upon stimulation of Auerbach's plexus in the gizzard; since the Auerbach's plexus is mostly nerve tissue, this evidence strongly supports the proposal that the adenine compounds came from nerves.

Tritiated adenosine uptake studies provide further evidence that ATP is the neurotransmitter (Su et al., 1971). Tritiated adenosine added to a medium in which strips of taenia coli were immersed was taken up by nerves and muscle to a considerable extent. Approximately 60 per cent of intracellular ^{3}H activity was ^{3}H-ATP. More of the ^{3}H-ATP activity was in nerve than in muscle. Little ^{3}H-ADP was formed; small amounts of ^{3}H-AMP (25%) and ^{3}H-inosine (10%) were present. Release studies were done on sections of taenia coli pre-incubated with tritiated adenosine. The medium contained atropine and guanethidine, thereby inhibiting

cholinergic and adrenergic nerves. Transmural stimulation resulted in an outflow of tritium activity into the medium; the activity could only have come from the intramural inhibitory nerves or the smooth muscle. Tetrodotoxin abolished both the muscle relaxation and tritium activity outflow normally resulting from intramural inhibitory nerve stimulation. Smooth muscle relaxation due to adrenergic nerve stimulation was not accompanied by tritium outflux. Therefore, the above evidence suggests that tritiated adenine compounds stored in intramural inhibitory nerves are released from the nerves during stimulation.

From the evidence, therefore, it can be concluded that nerves are present in the body which are neither adrenergic nor cholinergic. ATP seems to be the most probable candidate for the neurotransmitter substance of these nerves. The proposed purinergic nerves have thus far been implicated in the control of gastric, bladder and uterine motility through their effect on smooth muscle. Their function in organs such as the lungs is as yet unclear. The ATP released by these nerves seems to act by changing membrane premeability to potassium, with resultant changes in cell responsiveness.

Another group of compounds not discussed in this thesis but perhaps indirectly related to the pharmacology of the adenine nucleotides are the inosine, uridine, guanidine and cytidine nucleotides. The biological potency of some of these

compounds was reviewed by Drury (1936); and at that time the activity of these compounds in most preparations was considered to be less compared to the adenine compounds. However, cytidylic acid dilates the coronary vessels to about the same degree as adenylic acid (Wedd and Drury, 1934); and Flossner (1934) reported that members of the guanine series had a potency similar to the adenyl compounds. Hashimoto et al. (1964) found that uridine compounds produce vasodilation, UTP being somewhat less potent than ATP. Geiger (1956) has reported that a uridine compound produced in the liver is necessary for adequate perfusion of the brain. Wolf and Berne (1956) report that UTP has about the same effect as adenosine and AMP as a coronary vasodilator; however, these authors found that other derivatives of hypoxanthine, guanine, cytosine and uracil bases lacked vasodilator properties. Magnesium ITP was found to be only slightly less potent as a shock-inducing agent than magnesium ATP (Bielschowsky and Green, 1944).

Phosphate groups are readily transferable via kinases from one nucleotide to another (Beyer, 1968; Glaze and Wadkin, 1967; Goffeau et al., 1967); thus, even though a particular nucleotide is not biologically active, transfer of its phosphate may cause the formation of a nucleotide which can elicit a biological response. One can therefore imagine the possible extension of the physiological mechanisms proposed to be

mediated by ATP to include other nucleotides as well. Also, the possibility that different organs utilize different nucleotides as mediators should be considered.

2. Adenine Nucleotide Metabolism, General

The known pathways for the metabolism of the adenine compounds are diagramed in Figure 1. A review of the literature dealing with the metabolism of these compounds has revealed that the series of metabolic steps shown in Figure 1 is present in virtually all organs of the mammalian body. Some exceptions to the ubiquitious nature of these pathways may occur, however; and there is definite evidence that the quantitative importance of the alternative pathways shown in the figure may vary from one organ or tissue to another. The following sections devoted to individual organs and tissues describe our present understanding of these variations and also briefly describe the theoretical relationship of these pathways of adenosine metabolism to the local control of blood flow.

3. Adenine Nucleotide Metabolism in Whole Blood and Plasma

Drury et al. (1937) found that adenosine was inactivated by plasma of the cat and other species; AMP and ADP were also inactivated in plasma but less readily than adenosine. Following the studies of Drury, our understanding of the

Figure 1. Metabolism of the adenine compounds. 5'-nucleotidase = adenylate phosphatase = AMP phosphatase; apyrase = α-β-ATPase; adenylate kinase = myokinase = adenosine diphosphoric acid phosphomutase = adenosine triphosphate-adenosine 5'-monophosphate phosphotransferase; purine nucleoside phosphorylase = purine ribosyltransferase; hypoxanthine phosphoribosyltransferase = nucleotide pyrophosphorylase; adenine phosphoribosyltransferase = purine nucleotide pyrophosphorylase.

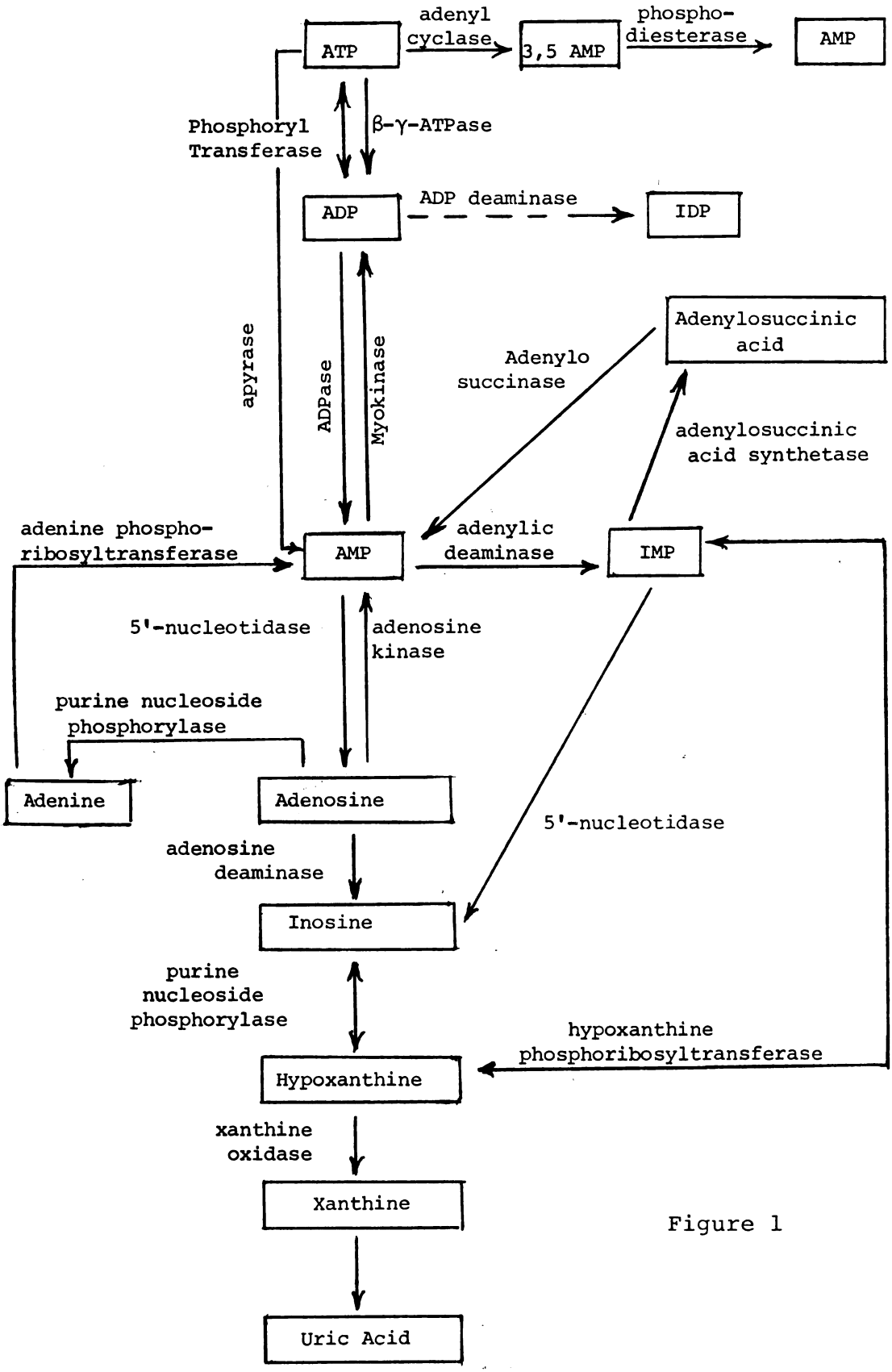
ATP
adenyl cyclase
3,5 AMP
phospho-diesterase
AMP
Phosphoryl Transferase
β-γ-ATPase
ADP
ADP deaminase
IDP
apyrase
ADPase
Myokinase
Adenylosuccinic acid
Adenylo succinase
adenylosuccinic acid synthetase
adenine phospho-ribosyltransferase
AMP
adenylic deaminase
IMP
5'-nucleotidase
adenosine kinase
purine nucleoside phosphorylase
Adenine
Adenosine
5'-nucleotidase
adenosine deaminase
Inosine
purine nucleoside phosphorylase
hypoxanthine phosphoribosyltransferase
Hypoxanthine
xanthine oxidase
Xanthine
Uric Acid

Figure 1

definitive pathways for metabolism of the adenine compounds by blood was initiated by the studies of Conway and Cooke (1939). These investigations indicated that the enzymes adenylic deaminase and adenosine deaminase are present in whole blood. Conway and Cooke also found these enzymes in plasma, but to a lesser degree than in whole blood. Purine nucleoside phosphorylase has been found in erythrocytes by Kim, Cha and Parks (1968). In regard to synthesis of nucleotides by blood, Miech and Santos (1969) have demonstrated the presence of adenosine kinase in erythrocytes, and adenylate kinase has been identified in erythrocytes by Tatibana, Nakao and Yoshikawa (1958) and Kashket and Denstedt (1958) and in platelets by Holmsen and Rozenberg (1968). The more indirect routes for nucleotide synthesis catalyzed by purine ribosyl- and phosphoribosyltransferase have been found in blood cells by Krenitsky (1969). The evidence for the presence of α-β-ATPase, β-γ-ATPase, ADPase and 5'-nucleotidase in plasma and platelets is discussed by Holmsen and Day (1971); and ATPase and apyrase have been reported to be in erythrocytes by Clarkson and Maizels (1952). Thus, virtually all the pathways for nucleotide metabolism have been identified in blood. The following paragraphs discuss additional findings that have been made regarding the metabolism of these substances in whole blood.

Early studies on stored human blood indicated that as the length of storage increased, the blood became less viable; cellular ATP levels fell and the normally discoidal erythrocytes became spherical. Jorgensen (1955) found that xanthine and hypoxanthine accumulated in stored human blood, apparently as a result of nucleotide degradation. Also upon storage, human blood became less viable after a time due to loss of acid-hydrolysable phosphate from nucleotides (Mills and Summers, 1959). Mills and Summers noted, in addition, that glucose and inosine were important in maintenance of cell viability, as cellular ATP levels increased when inosine or glucose was added to the blood. ATP is a required coenzyme in the conversion of glucose to glucose-6-phosphate, catalyzed by hexokinase, an important reaction for maintenance of cell viability. Guanine, cytidine, and 2,3-DPGA levels fell concurrently with a fall in ATP. Guanine and cytidine levels presumably fell as ATP may be necessary to maintain them. They suggested that the fall in 2,3-DPGA was contributing to the maintenance of the ATP level during storage of whole blood. Nakao et al. (1959) found that addition of adenine plus inosine to long-stored erythrocytes resulted in increased ATP levels and a return of characteristic disc shape to the erythrocytes, which had become geometrically distorted with aging. Neither adenine nor inosine added alone was effective in restoring ATP levels in long-stored erythrocytes. Inosine

was probably necessary to provide the ribose residue of the nucleotides and the glycolytic intermediates required for generating a high-energy phosphate supply, such as hexose monophosphate and hexose diphosphate.

Jorgensen and Poulsen (1955) found 1-3 µg/ml of hypoxanthine plus xanthine in the plasma of freshly withdrawn human blood. The concentration rose to 90-100 µg/ml upon standing at 37°C for 24 hours; intracellular ATP levels fell concurrently. There was also some increase at 4°C. Free phosphate liberation was slow, presumably due to its transfer to other substances. ATP levels also fell in stored human whole blood (Jorgensen, 1957). Hemolysis or stirring of the blood accelerated xanthine and hypoxanthine formation. ATP disappeared ten times faster in mechanically hemolysed blood as compared to non-hemolysed blood (Chen and Jorgensen, 1956). Chen and Jorgensen (1957) found that AMP conversion to hypoxanthine occurred at the same rate in hemolysed and non-hemolysed blood. A solution of erythrocytes in Tyrode-Locke solution also showed oxypurine (xanthine and hypoxanthine) accumulation. Leucocytes had no effect on oxypurine accumulation. There was no xanthine oxidase activity, as no uric acid was formed. Siliconized glassware reduced the rate of oxypurine formation.

These studies on stored whole blood therefore indicate that there is a continual degradation of ATP and simultaneous

formation of xanthine and hypoxanthine. In these experiments the specific intermediates between ATP and xanthine were not identified. Nonetheless, these initial findings with whole blood in conjunction with those of Conway and Cooke (1939) led to future investigation of the details of the metabolism of the adenine compounds in blood.

Jorgensen (1956) studied the breakdown of ATP in human platelet-rich plasma and whole blood. Nucleotide analysis was done by the spectrophotometric technique of Jorgensen and Chen (1956). ATP was added to whole blood or plasma in a concentration of about 0.4 mM. It was degraded to hypoxanthine in both plasma and whole blood. ADP, AMP, adenosine and inosine were identified as intermediates in this conversion. The half-time in plasma was approximately two hours. In whole blood the breakdown proceeded 7-8 times faster than in plasma, since the half-time here was approximately 1/2 hour. Since free phosphate concentration rose only half as rapidly as ATP was broken down, ATP breakdown was not one of simple ortho-phosphate liberation. Adenine added to plasma was not broken down, implying conversion of adenosine to hypoxanthine via inosine. The presence of adenosine deaminase, which converts adenosine to inosine, in plasma was thus implied; and nucleoside phosphorylase, which converts inosine to hypoxanthine, was also localized in plasma. The inosine-hypoxanthine conversion was found to be the rate determining step.

After eight hours at least 50 per cent of added inosine was converted to hypoxanthine with no decrease in the velocity of the reaction during this time. The reaction evidently did not reach equilibrium, as the velocity of hypoxanthine formation did not decrease in an eight hour period. Since hypoxanthine largely remained in the medium after its formation, ribose phosphate must have been continually removed to account for the maintenance of reaction velocity. The splitting of the ribose group from inosine by nucleoside phosphorylase was therefore the true determinant of the velocity of inosine breakdown to hypoxanthine in plasma.

Forrester (1969) studied the breakdown rate of ATP in diluted human plasma at room temperature. He found a 34 per cent fall in ATP concentration during the ten minute period following centrifugation of the blood. He also found that no ATP could be detected in plasma left standing at room temperature for one hour. Forrester (1972) further studied ATP breakdown in citrated human whole blood at 37°C. He found a linear relation between the amount of ATP added and the amount of ATP recovered from the plasma after centrifugation. Addition of low concentrations (1, 5, and 10 µg/ml) of ATP to whole blood at 37°C produced a consistent pattern of degradation with each concentration. For example, it took 32 minutes for 5 µg ATP/ml blood to be degraded beyond the threshold of detection, whereas 1 µg/ml required only about 12 minutes.

Holton (1959) found that 10 nmoles of ATP injected into rabbit ear arteries was both broken down in the blood and taken up by the tissues. Approximately 46 per cent of the adenine of ATP injected was not recovered in the venous effluent and was presumably taken up by the ear. Of the 54 per cent recovered, only 8 per cent was ATP; the remaining 46 per cent was ATP breakdown products.

ADP degradation in plasma has also been investigated. Holmsen and Stormorken (1964) found that an adenylate kinase reaction was the first step in ADP breakdown in human plasma, as both ATP and AMP were formed. AMP was inhibitory, while adenosine was not. However, Holmsen (1967) and Flatow et al. (1965) could not localize adenylate kinase in plasma. Plasma adenylate kinase activity found by earlier investigators was attributed to hemolysis. Holmsen and Stormorken (1964) found that EDTA in equimolar concentration with magnesium completely blocked ADP breakdown, while citrate had to be 18 times the magnesium concentration to be inhibitory. Pyrophosphate also inhibited breakdown, probably due to magnesium chelation. Heparin had no effect. Magnesium was required. Optimal pH was 8.5 to 8.8, although variation with substrate concentration probably occurred.

Odegaard et al. (1964) also studied ADP inactivation (removal of platelet clumping effect) in plasma. The inactivating system was heat labile and destroyed at 58°C. Maximal

activity was at 37°C. Dialysis had no effect and inactivation occurred in serum as well as plasma. AMP was formed.

ADP dephosphorylation to AMP by an ADPase in the plasma was found by Ireland and Mills (1966) to have a half-time of about 8 minutes. The K_m of the ADPase for ADP was 1-2 uM (Mills, 1966). Platelets did not enhance the breakdown. Adenylate kinase may also have been acting, as ATP was formed upon addition of 200 μM of ADP to platelet-poor plasma. Smaller quantities of added ADP, however, did not result in significant ATP accumulation. No IMP was detected. Use of citrate rather than heparin as anticoagulant resulted in approximately doubling the half-time of ADP (200 μM) breakdown in plasma; AMP also accumulated to a greater extent before disappearing. At lower initial ADP concentrations (1-2 μM), however, AMP accumulation differed little between heparin and citrate-treated samples. With heparin as anticoagulant more hypoxanthine accumulated in platelet-rich than in platelet-poor plasma.

The path of ATP breakdown in whole blood has been disputed. Mills and Summers (1959) found that IMP accumulated in the erythrocyte during an 8 hour incubation period. It was suggested that the IMP resulted from hypoxanthine anabolism rather than deamination of AMP, since IMP accumulated rapidly upon addition of inosine to the blood. Bishop (1960) found IMP formation in blood exposed to air and in hemolyzed

blood. The IMP was presumably formed from hypoxanthine in the presence of nucleotide pyrophosphorylase rather than by isosine formation, as inosine was not detected in stored or incubated blood. IMP was also presumed to be formed from the deamination of AMP. The equilibrium between IMP and AMP was in favor of IMP. Bishop therefore proposed that the pathway of ATP breakdown in whole blood proceeds via ADP, AMP, IMP and hypoxanthine. Ireland and Mills (1966), however, found no IMP formed during ^{14}C-ADP breakdown in plasma. In addition Conway and Cooke (1939) found that erythrocyte adenylic acid deaminase was normally inhibited due to the CO_2 and bicarbonate system of the blood. Also, Chen and Jorgensen (1957) found that addition of adenylic acid deaminase to blood caused a considerable change in intermediary compounds, suggesting that AMP deamination is a slow process normally. AMP dephosphorylation via 5'-nucleotidase seemed to occur much more rapidly. It was thus concluded that ATP breakdown in human blood takes place primarily via AMP, adenosine, and inosine to hypoxanthine.

Additional information concerning the breakdown path was found by Chen and Jorgensen (1957). IMP added to an erythrocyte suspension was converted to hypoxanthine two to three times as rapidly as AMP. This indicated that if AMP was deaminated to IMP, the resulting IMP could readily form inosine and hypoxanthine. Adenosine and inosine were converted to

hypoxanthine 50 to 75 times as rapidly as AMP. Therefore, adenosine deaminase acts more rapidly than 5'-nucleotidase in the erythrocyte. Added adenine resulted in no measurable hypoxanthine formation; therefore, it could not be an intermediate in ATP breakdown in whole blood unless it was formed in an isolated compartment.

The paths of ATP breakdown in whole blood and plasma described by Jorgensen (1956) and Bishop (1960) conflict, therefore, as to whether AMP is deaminated or dephosphorylated, Bishop's evidence suggesting deamination, while Jorgensen found evidence indicating dephosphorylation. Perhaps the concentration of AMP in part determines the pathway for its breakdown; the above discrepancies, therefore, may be caused by the different substrate levels studied by these investigators. Hemolysis and duration of the incubation are other factors which may affect the path of AMP breakdown in blood.

The enzymes involved in adenine nucleotide degradation in blood have been studied by many investigators. Mills (1966) found that 20 per cent of ATP added to human plasma was dephosphorylated to ADP by a plasma β-γ-ATPase, with a half-time of approximately 8 minutes, while a plasma α-β-ATPase dephosphorylated 80 per cent of the ATP directly to AMP with a half-time of about 3 minutes. The K_m of the α-β-ATPase for ATP was approximately 2×10^{-7}M. Increasing initial ATP concentration to 20 uM resulted in increased

formation of orthophosphate rather than pyrophosphate. Therefore, the α-β-ATPase appeared to be more easily saturated.

Parker (1970), in studies on human erythrocytes, found that ATP was degraded via ADP and AMP to adenosine by the outer surface of the erythrocyte membrane. Clarkson and Maizels (1952) isolated an apyrase from the stroma of human erythrocytes, which converted ATP to AMP. The apyrase had a pH optimum of about 6.8 and was stimulated by magnesium. Calcium had no effect. The apyrase acted on both extracellular and intracellular ATP. It is interesting to speculate that these enzymes in the erythrocyte membrane probably have some physiological significance related to membrane function.

Herbert (1956) also studied a human erythrocyte apyrase located on the outer surface of the membrane. This apyrase liberated orthophosphate from ATP and thus may be different from the apyrase* described by Clarkson and Maizels (1952). Herbert found that both calcium and magnesium activated his apyrase. The concentration range for magnesium was lower than that for calcium, however, possibly explaining why Clarkson and Maizels found no calcium effect. Calcium antagonized magnesium in a competitive manner. The pH optimum was 7.0 to 7.4. ADP was also dephosphorylated by the outer surface of erythrocytes. Caffrey et al. (1956) found a pH optimum of 7.3 to 7.6 for human erythrocyte ATPase.

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*It should be noted that the term apyrase refers usually to the enzyme producing AMP and pyrophosphate (rather than orthophosphate) from ATP (Caffrey et al., 1956).

Cysteine increased the enzyme's activity. ADP and ITP were also substrates, but with lower K_ms than ATP. A 1:1 ratio of magnesium to ATP was optimal for enzyme activity. The presence of carbon dioxide, bicarbonate, and phosphate in the erythrocyte reduced apyrase activity.

Adenyl cyclase, which converts ATP irreversibly to cyclic AMP, to the best of the author's knowledge, has not been found in dog erythrocytes (Davoren and Sutherland, 1963).

Conway and Cooke (1939) found adenylic acid deaminase, which converts AMP to IMP, in erythrocytes. The enzyme was normally inhibited, however, by the CO_2 and bicarbonate of the blood. They also found adenosine deaminase, which converts adenosine to inosine, in blood.

Baer et al. (1966) found and characterized adenosine deaminase in erythrocytes. The optimal pH was between 6.5 and 7.0. Schrader et al. (1972) located adenosine deaminase in both the cytoplasm and stroma of the human erythrocyte. The enzyme was evidently not on the outer surface of the membrane, however, since dipyridamole prevented adenosine deamination and uptake but did not interfere with adenosine deaminase activity.

Nucleoside phosphorylase, which reversibly splits the ribose from inosine to form hypoxanthine, was originally isolated by Kalckar (1945) from liver. Kim et al. (1968)

found nucleoside phosphorylase in high concentration in human erythrocytes. The enzyme had a broad range of optimal pH, from 6.5 to 8.0 and was quite stable.

The paths of adenine nucleotide synthesis in blood have also been investigated. The conversion of adenosine to AMP was studied by Miech and Santos (1969) in rat erythrocytes. They found that adenosine was converted to AMP without cleavage of the glycosidic bond, indicating the presence of adenosine kinase. Lowy and Williams (1966) also found direct phosphorylation of adenosine to AMP. Lerner and Rubinstein (1970) established the presence of adenosine kinase in erythrocytes and found that it was inhibited by ATP. Schrader et al. (1972) localized it in both the erythrocyte membrane and cytoplasm. Schrader et al. (1972) suggest that direct phosphorylation of adenosine occurs because the K_m of adenosine kinase is significantly lower than that of adenosine deaminase. Since both adenosine kinase and adenosine deaminase are present in erythrocytes, the fate of adenosine here is determined by the relative activities and K_ms of the two enzymes and the adenosine concentration.

Lowy and Williams (1966) found that the human erythrocyte is unable to convert IMP to AMP.

Bishop (1960) found adenine uptake by human erythrocytes. The adenine was then converted to AMP, presumably by reaction with ribosylpyrophosphate 5-phosphate catalyzed by nucleotide

pyrophosphorylase. Reaction of adenine with ribose-1-phosphate to form adenosine and thence AMP was considered unlikely due to high adenosine deaminase activity in blood; however, this may not be valid reasoning because low levels of adenosine rapidly go to AMP via adenosine kinase in blood.

ATP synthesis has been for the most part reported to occur intracellularly. Ronquist (1968) found extracellular ATP formed by human erythrocytes. However, the synthesis only occurred in a medium supplied with the substrates and cofactors of the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions. Tatibana et al. (1958) found a highly active adenylate kinase (myokinase) in human erythrocytes. This enzyme converted two molecules of ADP to AMP and ATP in a reversible reaction. Adenylate kinase activity was much higher than that of AMP deaminase. The enzyme was somewhat resistant to acid and quite resistant to heat. Flatow et al. (1965) found adenylate kinase activity in erythrocytes but not in leucocytes. Kashket and Denstedt (1958) isolated adenylate kinase from both the membrane and cytoplasm of rat and human erythrocytes. The presence of the enzyme in the cytoplasm permitted rapid interconversion of cellular nucleotides. Membrane-bound adenylate kinase believed located just below the surface could account for the conversion of extracellular ADP to intracellular ATP. ADP added to a suspension of washed erythrocytes appeared to enter

the external part of the erythrocyte membrane, whereas ATP or AMP apparently did not. The evidence suggested that the ATP or AMP formed from ADP could then pass freely into or out of the cell. AMP which entered the cells could evidently be converted to IMP, as large amounts of IMP were detected in stroma-free hemolyzate. Incubation of cells with ATP plus AMP resulted in ADP formation. Addition of AMP inhibited ATP formation from ADP. Clarkson and Maizels (1952) found that AMP phosphorylation could occur only within the erythrocyte membrane or intracellularly and required glucose.

It has been questioned as to whether de novo purine synthesis can occur in the erythrocyte. Evidence seems to indicate that it cannot. Lerner and Rubinstein (1970) found that adenine and glucose added to human erythrocytes resulted in increased intracellular ATP levels. The amount of the increase depended on the intracellular level of ATP prior to incubation. Addition of adenosine also resulted in increased intracellular ATP levels. Elevated ATP levels inhibited adenosine kinase. The maximum ATP level obtained with any cells or substrates was twice that found in fresh cells. Lowy and Williams (1966) also studied effects of adenosine and adenine on human erythrocyte ATP levels. They noted that adenine was only incorporated into ATP in the presence of inosine. Adenosine formed ATP at both high and low adenosine concentrations; however, due to the rapid deamination of

adenosine which occurred at low concentrations, much more ATP was formed at the higher concentrations.* Bishop (1960) found no evidence for de novo synthesis of purines from glycine in human erythrocytes. Lowy et al. (1958) could not find evidence for de novo purine synthesis in the rabbit erythrocyte.

Platelets contain large quantities of adenine nucleotides (Karpatkin and Langer, 1968). However, the role of platelet adenine nucleotides in the control of blood flow is not yet clear. Holmsen (1967) and Holmsen and Rozenberg (1968) found evidence for a metabolically inert ADP pool in platelets which was released in response to collagen, whereas a second ADP pool was metabolically active and not released by collagen. Karpatkin and Langer (1968) found considerable ATP generated with no increment in platelet ATP stores, implying a high rate of platelet ATP utilization. During the release reaction ATP goes irreversibly to IMP within the platelets and to hypoxanthine in the plasma (Ireland, 1967). AMP deaminase, therefore, appears to be much more important in platelet function than 5'-nucleotidase. Holmsen and Rozenberg (1968) found that adenosine was taken up by platelets to form ATP via AMP and ADP. Virtually no AMP was stored, and less

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*Another interpretation of this observation would be that more adenosine present at high adenosine levels caused more ATP formation by mass action. Schrader et al. (1972) found that adenosine kinase had a lower K_m than adenosine deaminase.

ADP was formed than ATP. ADP added to a suspension of washed platelets was converted to AMP extracellularly. Platelets enhanced hypoxanthine formation from ADP added to plasma (Ireland and Mills, 1964).

ATPases have been located on the surface of platelets (Chambers et al., 1967, and Mason and Saba, 1969). Chambers et al. (1967) characterized ecto-ATPase activity of human platelets. The enzyme was calcium and magnesium dependent, blocked by EDTA, and was maximally active in a pH range of 7.0 to 8.5. ADP competitively inhibited the enzyme. Ouabain had no effect, thereby differentiating this ATPase from the one involved in active transport of sodium and potassium. The enzyme was more active at 37°C than at 20°C. This ecto-ATPase may be the thrombosthenin described by Nachman et al. (1967). Thrombosthenin was characterized by its contractile properties and was implicated in clot formation and retraction, maintenance of platelet shape and ATPase activity. It was magnesium dependent and was located in both platelet membranes and granules. However, membrane thrombosthenin had greater ATPase activity than granule thrombosthenin. It resembled the protein which composes cellular microtubules, as it consisted of multiple polypeptide subunits of a polymeric nature.

Mason and Saba (1969) found that the platelet ecto-ATPase thrombosthenin was inhibited by sulfhydryl compounds.

Saba et al. (1969) isolated a light and a heavy platelet membrane fraction consisting of membrane bound vesicles. Thrombosthenin ATPase activity was associated with the light fraction.

Flatow et al. (1965) found adenylate kinase activity in platelets from an unspecified species, and Holmsen and Rozenberg (1968) found adenosine kinase in human platelets. Holmsen and Rozenberg also found that platelet lysates contained adenosine deaminase, AMP deaminase and purine nucleoside phosphorylase. Platelets were impermeable to AMP.

4. Adenine Nucleotide Metabolism in Skeletal Muscle

Conway and Cooke (1939) investigated adenylic acid deaminase in body tissues. Skeletal muscle had forty times more of the enzyme than other tissues, but the activity of the enzyme was 500-1000 times greater due to the absence of enzyme inhibitors in skeletal muscle. AMP was directly deaminated to IMP by this enzyme. Conway and Cooke found very low levels of adenosine deaminase compared to adenylic deaminase in skeletal muscle. Thus, in skeletal muscle the breakdown path of ATP differs somewhat from that occurring in whole blood and heart. In this regard Imai et al. (1964) noted a striking absence of adenosine in anoxic skeletal muscle; however, more recent reports from the same laboratory state that adenosine is normally present in skeletal muscle

and that its concentration increases with ischemic contraction (Dobson et al., 1971). In Imai's et al. studies there was a sharp drop in ADP during skeletal muscle ischemia which was not found in myocardial ischemia. IMP levels in skeletal muscle were also relatively high and increased shortly after ischemia due to ATP degradation and high adenylic acid deaminase levels. Creatine phosphate levels were 225 per cent higher in skeletal muscle than in cardiac muscle; and the authors provide evidence that this large pool of creatine phosphate in skeletal muscle helps maintain the ATP level in this tissue during ischemia. The IMP formed during ischemia was subsequently converted in these studies to inosine, presumably by a 5'-nucleotidase. Gerlach and Dreisbach (1962) found that the adenine nucleotide breakdown path in the rat kidney was similar to that in skeletal muscle, i.e., ATP, ADP, AMP, IMP, inosine and hypoxanthine.

Dobson et al. (1971) detected no ATP in the venous plasma from ischemic or normal skeletal muscle. However, no precautions (of significance*) were taken to prevent ATP breakdown during the isolation of the plasma. Adenosine, however, was found to increase fivefold in the venous plasma

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*Blood samples in these studies were cooled before centrifugation; however, plasma ATP degradation continues in whole blood at an appreciable rate even when iced; see Table 6, this thesis. Also, the samples were exposed to room air, which results in faster ATP breakdown than when physiological pH is maintained (see Figure 10, this thesis).

during ischemic contraction. Inosine and hypoxanthine increased 22 and 270-fold, respectively. It was therefore proposed that adenosine may regulate skeletal muscle blood flow in a manner similar to its role in coronary blood flow regulation; however, it was also concluded by these authors "that the adenine nucleotides are not directly involved in skeletal muscle blood flow."

Sutherland et al. (1962) localized adenyl cyclase in dog skeletal muscle and suggested that this enzyme was strongly influenced by circulating hormones. The role of adenyl cyclase here was presumably to influence cellular metabolism rather than to regulate blood flow.

Webster (1953) found that direct deamination of ADP by rabbit skeletal muscle myofibrils occurred, with resultant production of IDP and small amounts of IMP. Thus, in the skeletal muscle studies of Imai et al. cited above one must also consider the possibility of IDP formation. However, Webster also reports that as pH was increased from 5 to 7, dismutation of ADP was favored over deamination.

In regard to the synthesis of adenine compounds in skeletal muscle, Davey (1961) observed that AMP could be formed from IMP. The initial reaction involved a condensation of IMP and aspartic acid to form adenylosuccinic acid, catalyzed by adenylosuccinic acid synthetase. This was then converted to AMP. The pH optimum for this reaction was

between 6.9 and 7.2. GTP was the energy source and was found to be bound to sarcoplasmic proteins. Adenylosuccinic acid synthetase was detected in skeletal muscle and liver but not in heart, lung or kidney. The enzyme was implicated in the rapid deamination-reamination cycle of the adenine nucleotides proposed to occur during muscular contraction. Newton and Perry (1960) also studied the reamination of IMP in skeletal muscle. They noted that muscle potential for IMP reamination is less than for AMP deamination.

Kashket and Denstedt (1958) isolated adenylate kinase from skeletal muscle. The presence of the enzyme in the cytoplasm permitted rapid interconversion of cellular adenine nucleotides. Krenitsky (1969) found a high level of adenine phosphoribosyltransferase in skeletal muscle. This enzyme converts adenine to adenylate, which in turn could be converted to adenosine via 5'-nucleotidase or to ADP and ATP via adenylate kinase.

Inosine kinase, responsible for converting inosine directly to IMP, was characterized by Allan and Bennett (1971). The enzyme was isolated from E. coli. It required magnesium and was stimulated by potassium. It has not been found in mammalian tissues to the best of this author's knowledge.

Cell membranes have generally been considered to be impermeable to ATP. Chaudry and Gould (1970), however, found evidence that ATP may be taken up by rat soleus muscle.

^{14}

^{14}C-ATP added to soleus muscle was extensively degraded to ADP, AMP, and IMP. Small amounts of inosine and adenosine were also formed. AMP and IMP were found only extracellularly. Addition of ^{14}C-adenosine did not result in as much intracellular ^{14}C-ATP accumulation as did ^{14}C-ATP.* Addition of ^{14}C-ADP resulted in increased intracellular ^{14}C-ADP concentration. The evidence therefore suggested that rat soleus muscle was permeable to ATP, ADP and adenosine but not AMP or IMP. It was also determined that ATPase, adenylic acid deaminase and 5'-nucleotidase were located on the muscle surface, as their activities were not found in the incubation medium but the products of their reactions were. Also supporting nucleotide extrusion from the skeletal muscle cells, Boyd and Forrester (1968) found ATP released from exercising skeletal muscle. The authors argue that the ATP was not a result of damaged muscle cells, as potassium levels in the venous effluent did not rise; however, this conclusion is disputed by Dobson et al. (1971). Further consideration of this rather crucial proposal that these pharmacologically active adenine compounds can pass through the cell membrane is reviewed in more detail in a later section of this literature review.

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*This observation leads one to wonder if extracellular ATP can aid cellular adenosine uptake by supplying ATP for adenosine kinase in the cell membrane.

5. Adenine Nucleotide Metabolism in the Heart

The metabolism of the adenine compounds has been studied in the heart, perhaps to a greater degree than any other organ of the body. Evidence for the existence of several enzymes responsible for the metabolism of these substances in the heart has been published. Burger and Lowenstein (1967), Conway and Cooke (1939) and Baer et al. (1966) found adenylic deaminase and adenosine deaminase activity in this organ. Baer et al. (1966) also purified and studied 5'-nucleotidase in the rat heart. Colowick and Kalckar (1943) found low levels of adenylate kinase in rabbit heart muscle. Purine ribosyl- and phosphoribosyltransferases in the Rhesus monkey heart have been described by Krenitsky (1969). Cardiac muscle adenosine kinase activity was noted by Goldthwait (1957), and its purification has been reported by Alma and Feinberg (1971). Sutherland et al. (1962) found adenyl cyclase in the dog heart. Many investigations have been performed to determine the location and factors controlling the activities of certain of these enzymes in the heart and the theoretical relationship of their respective activities to the regulation of the coronary blood flow. The following discussion describes some of the observations and theories regarding these enzymes.

AMP is dephosphorylated to adenosine by 5'-nucleotidase. In the studies of Baer et al. (1966) optimal activity was at pH 9.5, and ATP was a powerful competitive inhibitor.

Since ATP levels fall during hypoxia, 5'-nucleotidase could be activated during this state, thereby increasing AMP breakdown to adenosine with resulting vasodilation rather than conversion to IMP. However, Burger and Lowenstein (1967) found that the fall in ATP during hypoxia did not appear to be sufficient to stimulate 5'-nucleotidase. AMP deaminase would presumably still be activated by the ATP levels present during hypoxia. However, adenosine formation may result from the increased rate of AMP formation during hypoxia or possibly a compartmentalization of 5'-nucleotidase, AMP deaminase and ATP. In the latter case ATP levels could conceivably fall low enough in localized areas or compartments of the membrane to activate 5'-nucleotidase. In regard to the intracellular localization of 5'-nucleotidase, Edwards and Maguire (1970) refer to unpublished studies of Maguire and Steggel, who found 5'-nucleotidase largely bound to cell membranes, i.e., the microsomal fraction of homogenized rat heart. The observations of Maguire and Steggel confirmed the similar findings reported earlier by Baer et al. (1966). Unlike Baer et al., the pH optimum reported by Edwards and Maguire (1970) for purified 5'-nucleotidase was 7.6 rather than 9.5 with AMP as substrate. Magnesium was found to induce shifts in the pH optimum, possibly explaining the different (9.5) pH optimum found by Baer et al. for their purified rat heart 5'-nucleotidase. Also, according to

Edwards and Maguire (1970) calcium inhibited this enzyme more strongly than magnesium; however, Baer et al. found 7 mM Mg activated and 7 mM Ca inhibited. ATP strongly inhibited rat heart 5'-nucleotidase in a competitive (Baer et al., 1966) and non-competitive (Edwards and Maguire, 1970) manner. Alkaline phosphatases also probably contributed to AMP hydrolysis in the studies of Baer et al., since their 5'-nucleotidase preparations hydrolyzed glucose-6-phosphate to a small extent. Burger and Lowenstein (1967) also have confirmed the finding that ATP inhibits "adenylate phosphatase" (5'-nucleotidase) from rat hearts.

Histochemical studies have also localized 5'-nucleotidase in the cell membranes of the rat heart (Bajusz and Jasmin, 1964, and Rostgaard and Behnke, 1965). Rostgaard and Behnke found AMP dephosphorylated in the interspace separating plasma membranes at the intercalated disks and at pinocytotic vessels but in no other myofibril structures but the T system. These observations have led Jacob and Berne (1960) to propose that 5'-nucleotidase in the cell membrane converts intracellular AMP to extracellular adenosine. Observations of others (Borgers et al., 1971) regarding the histochemical localization of 5'-nucleotidase in the dog heart indicate that this enzyme is more or less restricted to the perivascular and interstitial mesenchymal cell membranes of the heart. Thus, this latter author suggests that the Berne theory for regulation of coronary blood flow is not

valid since adenosine would be degraded to inosine by adenosine deaminase before it could get out of the cardiac muscle cell. Borgers et al. (1971) argue that adenosine from the perivascular cells controls the coronary blood flow. Berne currently has found 5'-nucleotidase in dog cardiac muscle membrane (in press) and thus substantiates his theory.

Another enzyme of key importance in the relationship between adenine nucleotide metabolism and the control of coronary blood flow is adenosine deaminase. By the conversion of adenosine to inosine the vaso-activity of the nucleoside is removed, and therefore the activity of adenosine deaminase could affect the coronary blood flow by influencing the cardiac adenosine levels. Its presence in virtually all tissues of the body was described by the Irish investigators Conway and Cooke (1939), and some of the properties of cardiac adenosine deaminase have been recently characterized. Baer et al. (1966) isolated and partially purified adenosine deaminase from rat hearts and noted that (1) its activity was solely in the supernatant cellular fraction; (2) its pH optimum was between 6.5 and 7.0; and (3) persantin 4×10^{-4} M, a coronary vasodilator, did not inhibit its activity. The finding that adenosine deaminase is in the heart tissue supernatant fraction does not necessarily indicate that this enzyme is present in the cardiac muscle cells; however, the low heart tissue concentration of adenosine may suggest that such

is the case. The loss of adenosine deaminase from the heart with Tyrode's solution perfusion is not easy to explain if adenosine deaminase is only intracellularly located (Jacob and Berne, 1960). Evidence that ATP and GTP do not alter the activity of rat heart adenosine deaminase has been reported by Burger and Lowenstein (1967).

It is rather difficult to propose a definite physiological role for adenosine deaminase in the heart. When ^{14}C-adenosine is infused into the isolated Tyrode-perfused cat heart, 50 per cent enters the adenine nucleotide pool of the heart and the remainder leaves the heart as ^{14}C-inosine and hypoxanthine in the perfusate (Jacob and Berne, 1960, and Jacob and Berne, 1961). Similar findings were made by Weidmeier, Rubio and Berne (1972) in the isolated perfused guinea-pig heart, and these authors state that they believe that virtually all of the adenosine entering the cardiac muscle cell is incorporated into nucleotides. Since adenosine seems to go into nucleotides rather than inosine in the heart, the biological role for adenosine deaminase in the heart is vague. The cardiac enzymes converting adenosine to AMP, ADP and ATP appear to be of greater importance even when the heart is anoxic (Jacob and Berne, 1961). Perhaps anatomically separate compartments of adenosine exist in the heart, and intra-arterially injected adenosine does not enter the adenosine compartment exposed to adenosine deaminase.

Rubio, Weidmeier and Berne (1972) state that the appearance of greater amounts of ^{14}C-isosine and ^{14}C-hypoxanthine in the perfusate than in the tissue of isolated ^{14}C-adenosine perfused hearts is an indication of the presence of adenosine deaminase in the capillary endothelium. In support of this hypothesis they refer to the finding of Conway and Cooke (1939) that adenosine deaminase is present in a non-specified artery. In regard to this rationalization for the low adenosine levels found in coronary venous blood, the fact that adenosine deaminase and nucleoside phosphorylase are found in the perfusate of isolated perfused hearts (Jacob and Berne, 1960) should at least be considered. Should these enzymes have been present in the perfusate of the isolated perfused hearts of Rubio, Weidmeier and Berne (1972), it is quite obvious that considerable amounts of ^{14}C-adenosine could have been converted to inosine and hypoxanthine directly in their perfusates. In order to eventually determine and clearly understand the mechanisms for the chemical control of an organ's blood flow, it may be best not to favor any one single theory at this time. Indeed, the selection and discussion of data only in terms of its support of one's pet theory may be detrimental.

The observation described in the previous paragraphs that exogenous ^{14}C-adenosine enters primarily adenine nucleotides in heart tissue brings focus upon the enzymes responsible

(primarily adenosine kinase) for permitting such conversion. Although several enzymes to be discussed later can influence the conversion of adenosine to nucleotides, adenosine kinase is of primary importance because it directly converts adenosine to AMP. The presence of adenosine kinase has been shown in dialyzed pig heart homogenates by Goldthwait (1957) by demonstrating the capacity of such preparations to rapidly convert ^{14}C-adenosine to AMP in the presence of ATP. Sister Alma and Feinberg (1971) characterized cardiac muscle adenosine kinase. Magnesium enhanced its activity but was not required. Optimal pH was 5.8. Dipyridamole had no effect on adenosine kinase activity. Low ATP concentrations (2-8 x 10^{-4} M) stimulated the enzyme while higher concentrations (> 8 x 10^{-4} M) inhibited. Thus, cardiac anoxia with low cellular ATP levels may enhance the conversion of adenosine to AMP in spite of lower levels of ATP as a substrate for adenosine conversion to AMP. Indeed, the studies of Jacob and Berne (1961) seem to confirm this possibility. Lindgerg et al. (1967) found that activity of adenosine kinase decreased with time at 37°C and pH 7.4.

Other enzymes can also participate in the conversion of adenosine to the adenine nucleotides. Should adenosine be converted to adenine in the heart, cardiac adenine phosphoribosyltransferase (Krenitsky, 1969) could act on the adenine to form AMP. Another alternative would be for adenosine to

be deaminated to inosine by adenosine deaminase and subsequently converted to hypoxanthine and IMP by the cardiac enzymes nucleoside phosphorylase (Rubio, Weidmeier and Berne, 1972) and hypoxanthine phosphoribosyltransferase, respectively, as indicated in Figure 1. The quantitative insignificance of these enzymic pathways for conversion of adenosine to nucleotides in comparison to the adenosine kinase step has been demonstrated by Goldthwait (1957) in the pig heart and Weidmeier et al. (1972) in the isolated, perfused guinea-pig heart. The lack of conversion of adenosine to adenine in the rabbit heart was reported by Liu and Feinberg (1971) on the basis of conversion of adenosine-8-^{14}C to nucleotides without formation of ^{14}C-adenine. However, Jacob and Berne (1960) report adenine formation from ^{14}C-adenosine in the isolated, perfused cat heart. In spite of their relative insignificance in adenosine metabolism, the presence of these alternate routes for nucleotide synthesis has been shown by Goldthwait (1957) in the heart and may indicate that the nucleotide pool in the heart is in a dynamic state with continual breakdown and resynthesis occurring. The construction of the metabolic scheme for these events may involve compartmentalization of the different enzymes; and within a tissue or organ synthesis and degradation of the nucleotides conceivably could occur both in the same or different sites. Thus, exogenous adenosine may be directed to adenosine kinase, whereas some endogenous

adenosine may possibly be converted to adenine or hypoxanthine before conversion to AMP.

The proposed dynamic state of the cardiac nucleotides alluded to in the previous paragraph may also involve the interconversion between AMP and IMP, as indicated in Figure 1. The enzyme adenylic deaminase, which converts AMP to IMP, is certainly present in heart tissue (Goldthwait, 1957; Conway and Cooke, 1939; and Baer et al., 1966). In rat heart homogenates Baer et al. (1966) found adenylic deaminase in nuclear, mitochondrial, microsomal and supernatant fractions; its activity was mostly found in the nuclear and supernatant fractions. Burger and Lowenstein (1967), using a semi-purified preparation of rat heart adenylic deaminase, demonstrated that ATP activates and GTP inhibits this enzyme. Thus, with decreased ATP in the heart AMP conversion to adenosine rather than IMP could be favored due to decreased adenylic deaminase activity and increased 5'-nucleotidase activity in localized areas. These findings therefore support the Berne theory that increased adenosine production and release during anoxia cause cardiac vasodilation. However, these findings also bring into focus the criticism that studies concerning the quantitative importance of adenylic deaminase as compared to adenosine deaminase (Conway and Cooke, 1939) must be reviewed with consideration of the ATP level in the enzyme preparation.

In studies of adenine nucleotide degradation in the isolated perfused rabbit heart by Richman and Wyborny (1964), it

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was suggested that the principle path for AMP degradation is initial dephosphorylation to adenosine. The basis for this conclusion was the appearance of inosine and hypoxanthine in the perfusate concomitant with nucleotide breakdown triggered by anoxia and uncoupled oxidative phosphorylation. Nonetheless, since IMP increased in the cardiac tissue with uncoupling, the authors indicate that direct adenylic acid deamination must at least be operative. It is difficult to understand why Richman and Wyborny (1964) did not find cardiac tissue IMP in their controls. With high tissue ATP in the control state adenylic deaminase should be most active; perhaps low control tissue AMP did not permit IMP formation. One also wonders if the increased IMP formed during anoxia is the cause of increased inosine in the venous effluent during hypoxia reported by Berne (1963), since IMP like AMP is a substrate for 5'-nucleotidase.

As indicated in Figure 1, adenylosuccinic synthetase and adenylosuccinase can convert IMP to AMP. To the best of our knowledge these enzymes have not been identified in the heart; however, their possible existence in this tissue has been discussed by Weidmeier, Rubio and Berne (1972). On the basis of the observation that the specific activity ratios of nucleoside:base in heart perfusion fluid were the same as heart tissue nucleotides, these authors conclude "that the degradation of adenosine to base prior to its incorporation

into adenine nucleotides is minimal". Again, however, one must consider the possibility that exogenous adenosine may not reach the sites for its enzymic degradation.

The adenosine diphosphate deaminase reported by Webster (1953) to exist in rabbit skeletal muscle fibrils was not found in rabbit heart homogenates, actin or myosin.

Adenyl cyclase, localized in the dog heart by Sutherland et al. (1962), converts ATP to cyclic AMP. The enzyme in heart tissue was very sensitive to catecholamines; an increased catecholamine level stimulated adenyl cyclase. The primary function of this enzyme in the heart, therefore, is probably to increase cellular metabolism and thus support an increased force of contraction. No direct role of adenyl cyclase in the regulation of coronary blood flow was found.

The intracellular and intratissue location of one of the major enzymes for nucleoside degradation has been studied by Rubio, Weidmeier and Berne (1972). These investigators, using a new histochemical method for nucleoside phosphorylase, observed this enzyme in the cytoplasm and nuclei of endothelial cells and pericytes but not in the myocardial or smooth muscle cells. These findings apparently explain the appearance of relatively large quantities of hypoxanthine in the perfusate of the isolated heart compared to the tissue itself. It should be considered, however, that the hypoxanthine could arise directly from interstitial inosine as well

as indirectly from interstitial adenosine as suggested by the authors. If adenosine deaminase and nucleoside phosphorylase are in the capillary endothelium, one wonders why adenosine entering the isolated, perfused heart does not become completely degraded to hypoxanthine before it reaches the cardiac cells. Should Schrader's proposal that the low K_m of adenosine kinase causes adenosine phosphorylation rather than deamination apply to the heart as it does for the erythrocyte ghost, then adenosine leaving the cardiac cell should be rapidly reincorporated into AMP rather than being washed out of the heart as hypoxanthine.

As mentioned previously, Berne has proposed that adenosine may be the chemical mediator of coronary vasodilation in the hypoxic heart (Berne, 1963). 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 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 arterial blood or in venous blood from a well-oxygenated heart. Berne proposed,

therefore, that during hypoxia the decreased oxygen tension caused breakdown of heart muscle adenine nucleotides to adenosine. The adenosine then would diffuse out of the cells and reach the coronary arterioles 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 and washing out high levels of adenosine. The feedback mechanism would thus provide a way to adjust coronary flow to meet the heart's oxygen needs.

Richman and Wyborny (1964) found evidence to support Berne's theory. Adenosine was recovered in the perfusate during adenine nucleotide degradation in the heart resulting from hypoxia or uncoupled oxidative phosphorylation. The study showed that adenosine can leave cells of the heart in the presence of either adenosine deaminase saturation or inhibition with 8-azaguanine, since adenosine appeared in the perfusate under these conditions. Large quantities of inosine were recovered in spite of adenosine deaminase inhibition by 8-azaguanine. Therefore, the direct deamination of AMP is an operational pathway in the intact heart, although these investigators suggest that it is not the one used normally. Further evidence in support of Berne's theory comes from the studies of Imai et al. (1964), who under conditions of complete ischemia found adenosine in the underperfused

myocardium of the rabbit. In addition, Chen et al. (unpublished observation) found that the ischemic myocardium released a substance which vasoconstricted a bioassay kidney; adenosine injected into the kidney was found to have the same effect. Therefore, adenosine perhaps was in the venous effluent of the ischemic myocardium. Katori and Berne (1966) found adenosine in the perfusate of hypoxic, anoxic or epinephrine-treated cat hearts in the presence of 8-azaguanine. Graded hypoxia showed that increased coronary flow was roughly proportional to the sum of adenosine, inosine and hypoxanthine released (Wiedmeier et al., 1970).

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 indicated that interstitial fluid adenosine levels could regulate coronary blood flow to maintain a proper oxygen balance. Rubio et al. (1969) found that the amount of adenosine released during short periods of reactive hyperemia in the dog heart was sufficient to account for observed dilation. The adenosine was believed to accumulate only in the extracellular space of the ischemic myocardium, because heart tissue adenosine content was quantitatively accounted for by an estimation of interstitial fluid adenosine.

6. Metabolism of Extracellular Adenine Nucleotides by the Lungs

Folkow, as early as 1949, reported that intravenously injected ATP was inactivated (its hypotensive action removed) upon passage through the lungs. This conclusion was based on the observation that a much greater injection of ATP was required to lower systemic arterial pressure when injected intravenously than when injected intra-arterially. Brashear and Ross (1969) found that ADP passed through the pulmonary circuit without complete breakdown, since intravenously injected ADP was recoverable in arterial blood. Gordon (1961), repeating Folkow's experiments, found that ATP and adenosine were inactivated in the lungs; however, adenosine inactivation was to a lesser extent. According to Gordon ADP and AMP were not subject to this pulmonary inactivation. Pfleger et al. (1969) found that 57 per cent of ^{14}C-aenosine intravenously administered was taken up by the lungs in 30 seconds. Inosine addition reduced this uptake. The importance of organ uptake was shown by estimates which suggested that more than 90 per cent of injected adenosine was taken up by organs and that only 10 per cent was broken down by the blood. Afonso and O'Brien (1971) found that dipyridamole greatly decreased uptake of adenosine by organs, especially the lungs, suggesting in view of Schrader's red cell ghost model that adenosine enters the lung and other

tissues of the body via facilitated diffusion. A comparison of adenosine disappearance rates in different tissues showed the following: .76 ug/min/ml in blood; 156 ug/min/gm in heart; and 2117 ug/min/gam in lung. Clarke et al. (1952) showed that the adonosine deaminase content of the lungs was very high; and Burger and Lowenstein (1967) identified both adenylic and adenosine deaminase in lung tissue. Considering the avid metabolism of adenosine by the lungs, this organ may therefore function to provide the body with arterial plasma free of adenosine, as well as fulfilling its more publicized gas exchange and angiotensin II formation functions. Of course, the validity of this speculation regarding adenosine hinges on whether or not adenosine and/or adenine nucleotides exist physiologically in venous plasma, and whether or not the blood itself is capable of rapidly destroying these pharmacologically active compounds.

Another source of adenine compounds in the lungs may be the non-adrenergic inhibitory nerves supplying the interstitial smooth muscle identified by Robinson et al. (1971) in the toad lung. Relaxation of the lung upon vagal stimulation was unaffected by adrenergic and cholinergic blocking agents. Although the neurotransmitter in this case has not yet been identified, Burnstock et al. (1970) found evidence that ATP is the transmitter released by similar nerves in the gut.

7. The Passage of Adenosine and Adenine Nucleotides Through the Cell Membrane

The ability of adenosine to pass through cell membrane is well documented. According to Rubio, Berne and Katori (1969), adenosine passes out of the cardiac muscle cells into the blood perfusing the heart. Regarding the movement of adenosine in the opposite direction, Su et al. (1971) have found ^{3}H-adenosine taken up by enteric nerves in considerable quantities. The ^{3}H-adenosine was rapidly metabolized in the nerve, largely to ^{3}H-ATP. Jacob and Berne (1960) demonstrated the uptake of adenosine by the isolated perfused heart, and Schrader et al. (1972) have provided evidence that ^{14}C-adenosine rapidly enters the red cell ghost. In the red cell ghost adenosine uptake had two components: a facilitated diffusion system which saturated at about 10 mM and a simple diffusion system which did not saturate. At low extracellular adenosine concentrations (below 3 uM), most of it was taken up by facilitated diffusion and converted to adenine nucleotides in the erythrocyte. At higher concentrations there was much degradation to inosine and hypoxanthine, since the K_m of adenosine deaminase was much higher than that of adenosine kinase. Adenosine was found intracellularly only when external concentrations were as high as 1 mM. Intracellular adenosine metabolism was so fast that a concentration

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gradient from out to in would exist under physiological conditions. Adenosine uptake would therefore occur whenever extracellular adenosine was present. Berne suggests that in the anoxic heart outflux rather than uptake of adenosine occurs--a theory which may conflict with the proposal of Schrader.

Parker (1970) found that the adenosine formed from ATP degradation in blood did not accumulate but was rapidly taken up by the erythrocytes and rephosphorylated directly to AMP. The adenosine uptake showed kinetics of a saturable carrier transport system, in agreement with Schrader et al. (1972). The purine portion of the adenosine taken up by the erythrocyte was homogenously distributed through the cell adenine nucleotide pool.

Extrapolation of erythrocyte data to cardiac muscle indicates that at physiological concentrations, adenosine enters the cells by facilitated diffusion, since such concentrations extracellularly would be much less than 10 mM. In support of this proposal the uptake of adenosine by the heart and its incorporation into the adenine nucleotide pool of the heart is well documented (Jacob and Berne, 1960, and Weidmeier, Rubio and Berne, 1972). Thus, it is undoubtedly evident that adenosine is capable of passing through the membranes of the

cardiac cells. However, the actual mechanism by which adenosine enters the cells of the heart, like other tissues of the body, has not been determined. Nonetheless, in this latter regard the studies of Afonso and O'Brien (1971) have demonstrated that both dipyridamole and lidoflazine greatly reduce the uptake of adenosine by the dog heart. Since these drugs did not reduce the destruction of adenosine by myocardial homogenates, it appears that they reduce cardiac adenosine uptake by decreasing the permeability of the cells to this nucleoside. These findings may be indicative of a carrier-mediated transport mechanism for adenosine in the heart similar to that proposed by Schrader, Berne and Rubio (1972) for the red cell ghost.

A relationship between red cell adenosine uptake and red cell deaminase activity has been described by Van Belle (1969). He found that both platelets and erythrocytes were responsible for the disappearance of adenosine in the dog and guinea pig. Ireland and Mills (1966) also found that platelets were permeable to adenosine. However, in human blood Van Belle found that the erythrocytes were more important in regard to adenosine uptake and deamination. Variations in pH between 6 and 8 and ions did not affect adenosine uptake or deamination in dog or human blood. Increasing temperature from 20°C to 35°C resulted in increased adenosine uptake and

deamination in both human and dog blood. Use of metabolic inhibitors showed no dependence of adenosine uptake on general metabolism, glycolysis, oxidative phosphorylation or transport ATPase. Adenosine uptake therefore appeared to depend on intracellular adenosine activity unless the cell membrane was changed in a way which altered sulfhydryl groups. Membrane sulfhydryl group integrity was necessary for normal uptake.

Evidence for rapid entry of adenosine into red cells has also been reported by Whittam (1960). Whittam studied uptake of inosine, adenosine, hypoxanthine and adenine by human erythrocytes. Inosine was taken up in direct proportion to its concentration in the medium. Equilibrium between cells and medium was reached within five minutes. Adenosine was also rapidly taken up by erythrocytes and deaminated to inosine. Less formation of nucleotides occurred, probably because of the high levels of adenosine used by Whittam. Hypoxanthine, regardless of the initial concentration in the medium, reached equilibrium across the red cell membrane; and no appreciable concentration was established between cells and medium. Temperature, glycolysis, or the presence of similar compounds had no effect on uptake. Adenine in low concentrations, in the presence of a substrate for glycolysis, was avidly taken up by erythrocytes at 37°C. Only a small amount (12%) of the adenine taken up leaked back into the

medium. At high adenine concentrations (3-7 mM) glycolysis had no effect on uptake. Adenine was presumably converted into adenine nucleotides within the erythrocyte, since iodoacetate blocked glycolysis and also significantly decreased ^{14}C-adenine uptake. In addition, iodoacetate added after 30 minutes incubation resulted in very little leakage of intracellular ^{14}C-adenine or its derivatives back into the medium. Therefore, the derivatives of the adenine may have been adenine nucleotides, as these compounds are presumably non-diffusable through cell membranes. The adenine uptake was not due to active transport, based on this evidence. Bishop (1960) also found adenine taken up by human erythrocytes. Henderson and LePage (1959) found that adenine-8-C^{14} was taken up by mouse erythrocytes in vitro. This adenine was lost in some measure to body tissues upon injecting the blood into an animal. Hamilton (1953) found adenine-8-C^{14} taken up by human leucocytes.

The passage of adenine nucleotides through cell membranes is less well documented and indeed, some investigators (Jacob and Berne, 1960, Lowry, Romot and London, 1958, and others) have stated that the cell membranes of various tissues are relatively impermeable to these phosphorylated compounds. Ireland and Mills (1966) presented evidence that platelets were impermeable to ADP and AMP. Nonetheless, studies of others indicate that nucleotides may escape in at least small

amounts from the intracellular to the extracellular fluid. Forrester (1972b) and Chen et al. (1972) report values for ATP and AMP in plasma. Abood et al. (1962) report efflux of ATP from muscle and nerve and relate this phenomenon to depolarization. The release of ATP from non-adrenergic inhibitory nerves in the gut was noted by Burnstock et al. (1970) and Su et al. (1971). They also found uptake of tritiated AMP, ADP and ATP by these nerves but to a lesser extent that of tritiated adenosine. However, the possibility that the nucleotides were first converted to adenosine before uptake was not considered. Kuperman et al. (1964) found tritiated AMP released from frog sciatic nerve; they also stated that ATP behaves similarly. Chaudry and Gould (1970) report evidence for the direct entry of ATP and ADP into the rat soleus muscle; whereas, Hoffman and Okita (1965) report that the penetration of ATP into the myocardium is initiated and takes place through ATP breakdown followed by intracellular resynthesis. Conway and Cook (1939) stated that AMP could readily pass through the red cell membrane and 'voluntary muscle'; however, we could not find the authors' experimental evidence for these statements. Levine et al. (1969) found that rat liver cells were slightly permeable to exogenous cyclic adenosine 3',5'-monophosphate-8-^{14}C, and Davoren and Sutherland (1963) found extracellular cyclic AMP accumulation following its intracellular synthesis. Douglas et al. (1966)

found a high ATP concentration in the chromaffin granules in adrenal medulla cells and described evidence for its release into the extracellular fluid. Apparently, ATP and other adenine nucleotides were discharged into the blood vessels along with catecholamines upon medullary stimulation; however, ATP was quickly degraded to AMP in the adrenal blood vessels.

Taken as a whole the studies described above indicate the possibility that small quantities of adenine nucleotides may leak out of the cells of the body. If such is the case, the large number of pharmacological effects of extracellularly administered adenine nucleotides which have been listed in Table I may have some physiological significance. It thus becomes of prime importance and one of the objectives of this thesis to re-examine the possibility of outflux of intracellular adenine nucleotides.

The studies described in this literature review indicate a need for further investigation of the role of adenine compounds in the body. It should be determined whether or not they exist in normal arterial plasma, affect respiration under physiological conditions, and/or mediate hyperemia. The studies presented in the Results of this thesis are a preliminary attempt to answer and formulate approaches to these questions by investigating adenine compound metabolism in blood and during passage through the lungs.

METHODS

1. Breakdown of ^{14}C-ATP in Plasma

Male and female mongrel dogs of both sexes were obtained from C.L.A.R. and anesthetized with 25 mg sodium pentobarbital per kg body weight intravenously. The femoral artery was cannulated with polyethylene tubing; and approximately 10 ml of arterial blood was collected into a siliconized tube which contained 100 units of heparin. The blood was immediately centrifuged twice in a Sorvall centrifuge at 10,000 x G for 10 minutes each time. Two 2 ml platelet-free plasma samples were then pipetted into siliconized test tubes. A 50 λ sample of ^{14}C-ATP (1 μCi; 1150 ng ATP) was added to each. The plasma was then incubated in a constant temperature water bath at 37.5° C for a selected incubation time. One-half ml of carrier nucleotides (60 mg ATP, 25 mg ADP, 20 mg AMP and 5 mg adenosine) were added to each sample in order to be able to determine subsequently by optical density measurements the specific fraction collector sample tubes containing each nucleotide. The plasma, with carrier nucleotides added, was then poured directly onto a column of Dowex 1 resin, .9 cm in diameter and packed to 6.2 cm, and a gradient elution with ammonium formate immediately commenced.

2. Breakdown of ^{14}C-ATP in Whole Blood

Approximately 10 ml of arterial blood was collected into a siliconized tube containing 100 units of heparin. After gently swirling the blood, two 2 ml samples were pipetted with siliconized pipettes into siliconized test tubes and allowed to equilibrate in a 37.5° C constant temperature water bath for one to two minutes. A 50 λ sample of ^{14}C-ATP (1 μC; 1150 ng ATP) was then added to each and the mixture incubated for an accurately measured time. Seven ml of 6% perchloric acid was added to each sample at the end of the incubation period to denature proteins and stop ATP breakdown. One-half ml of carrier nucleotides was added to each sample. The samples were then centrifuged at 10,000 x G for 10 minutes at -3° C for denatured protein removal. The supernatants were decanted into test tubes and placed in an ice bath. Perchloric acid was removed by titration with 5 M potassium carbonate, using methyl orange as the end point indicator. The protein-free supernatant was then poured directly onto a column of Dowex 1 anion exchange resin for nucleotide separation by gradient elution ion exchange chromatography. Hematocrit and pH were determined on the excess blood which was carried through identical incubation procedures.

3. Separation of Adenine Nucleotides by Ion Exchange Chromatography

A gradient elution ion exchange chromatographic apparatus similar to that described previously by Busch et al. (1952)

for organic acids was used to separate the adenine nucleotides. Similar procedures for separating nucleotides have been described by Egawa and Neuman (1964), Cohn and Carter (1950), Cohn and Bollum (1961), Cohn (1950), Hurlbert et al. (1954), Blattner and Erickson (1967) and Burger and Lowenstein (1967). Problems related to reaction of ATP with the anion exchange resin utilized in these procedures have been described by Lund et al. (1954); however, to the best of our knowledge such chemical alteration of ATP did not occur in our procedures since both carrier and ^{14}C-labeled ATP were completely recoverable from the resin columns.

The procedures modified from those reported above which were used in the studies described in this thesis were as follows. 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 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 .9 cm inner diameter packed with 6.2 cm of Dowex 1 anion exchange resin in the formate form. 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 or more 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 chromatographic separation is shown in Figure 2 and the pattern of nucleotide elution is shown in the upper diagram of Figure 3. In certain experiments an elution with distilled water for the first 30 fraction collector tubes before adding 1 M ammonium formate was used to more completely separate the nucleosides from nucleotides.

Since it was observed that carrier IMP and AMP came off the resin in the same fraction collector samples with the above procedures, a gradient elution procedure utilizing formic acid was modified from that of Hurlbert et al. (1954) to separate AMP from IMP. The details of the elution pattern

Figure 2. Gradient elution ion exchange column chromatography apparatus.

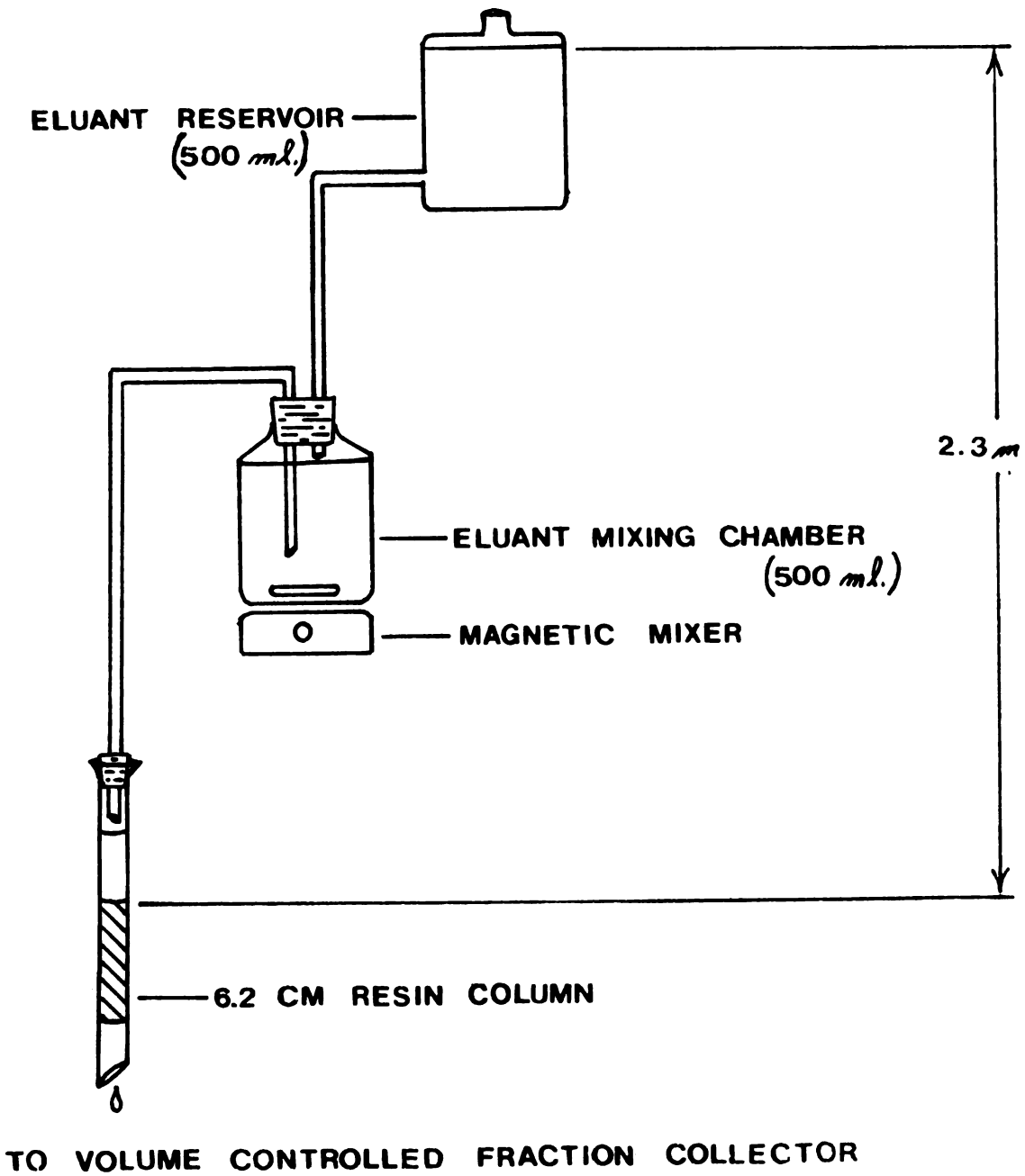
ELUANT RESERVOIR
(500 ml.)
2.3 m
ELUANT MIXING CHAMBER
(500 ml.)
MAGNETIC MIXER
6.2 CM RESIN COLUMN
TO VOLUME CONTROLLED FRACTION COLLECTOR

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 mu. 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.

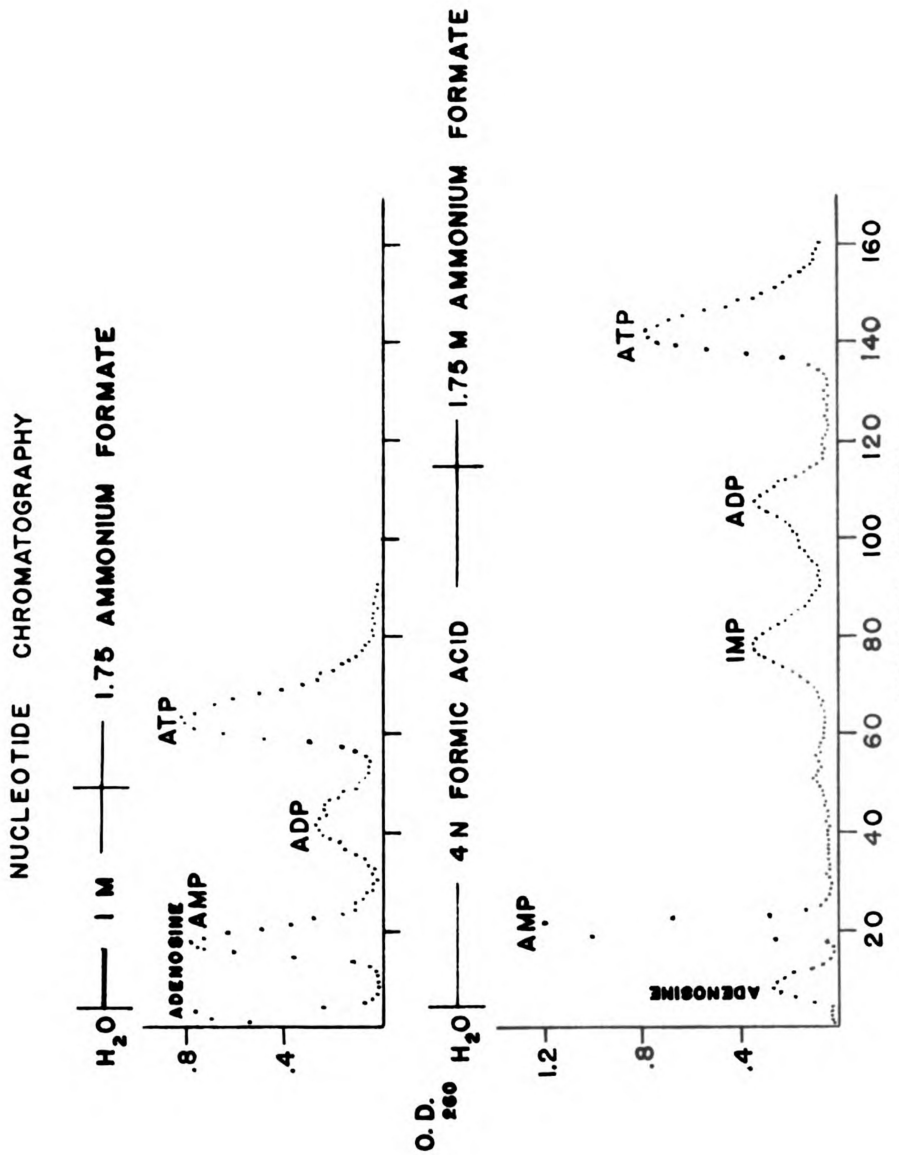
NUCLEOTIDE CHROMATOGRAPHY
H2O
1 M
1.75 AMMONIUM FORMATE
ADENOSINE
AMP
ADP
ATP
.8
.4
O.D. 260
H2O
4 N FORMIC ACID
1.75 M AMMONIUM FORMATE
AMP
ADENOSINE
IMP
ADP
ATP
1.2
.8
.4
20
40
60
80
100
120
140
160
SAMPLE NUMBER

Figure 3

for this procedure are described in Figure 3 (lower diagram) and Section 7 of Methods.

4. ^{14}C-ATP Purity Check

A purity check was done on the ^{14}C-ATP by adding a 50 λ sample of the isotope in 2 ml of saline with 0.5 ml of carrier nucleotides to the resin column described above and chromatographically separating the ATP. 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 Baird Atomic 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. The results of a purity check on the ^{14}C-ATP used in the present studies is shown in Figure 4. Ninety-three per cent of the total ^{14}C activity in the sample was ^{14}C-ATP. ADP, AMP, and nucleosides each accounted for approximately 2% of the total ^{14}C activity added to the resin column.

5. ^{14}C-Adenosine Purity Check

One μCi of ^{14}C-adenosine in 1 ml of saline with 0.5 ml of carrier nucleotides was chromatographically separated as described above. Identification of adenine compounds present was inferred by comparison of ^{14}C-labeled peaks with carrier

Figure 4. ^{14}C-ATP and ^{14}C-adenosine purity checks. ^{14}C-ATP (1 µCi) or ^{14}C-adenosine (1 µCi) were added to Dowex 1 ion exchange resin and eluted with 1 M ammonium formate to sample #50; 1.75 M ammonium formate to sample #90. One ml of each sample was then plated, dried, and counted. The separation pattern of the adenosine compounds for this chromatography procedure is shown in the upper diagram of Figure 3.

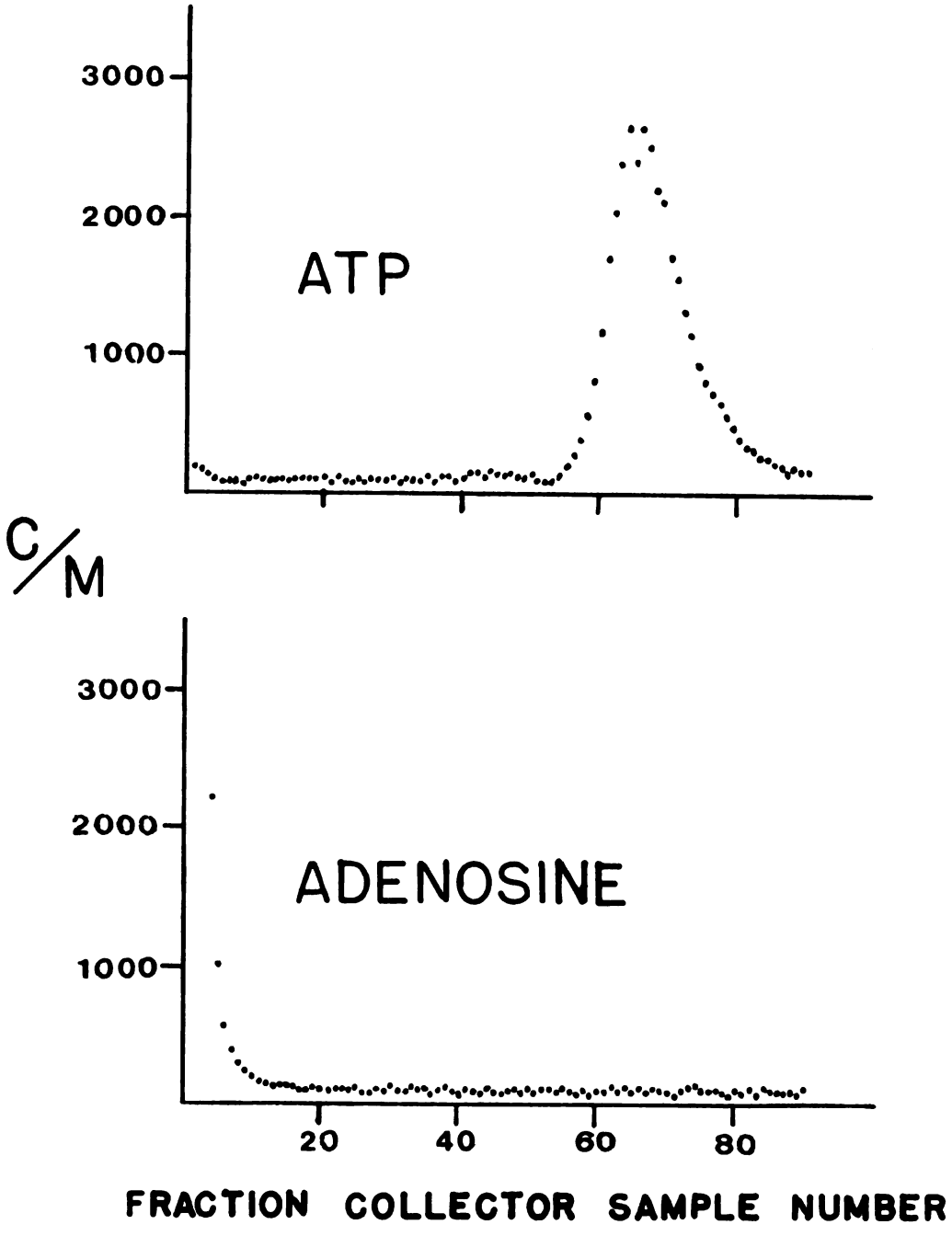
3000
2000
1000
ATP
C/M
3000
2000
1000
ADENOSINE
20
40
60
80
FRACTION COLLECTOR SAMPLE NUMBER

Figure 4

nucleotide peaks. The results of one ^{14}C-adenosine purity check are presented in Figure 4.

Since adenosine, inosine, and hypoxanthine were all eluted with distilled water in the first fraction collector samples, it was not possible to differentiate individual nucleosides with the separation procedure used. Calculation of nucleoside ^{14}C activity as per cent of total showed that 95% of total ^{14}C activity was clearly nucleosides. The remaining 5% of the counts was distributed in the areas of AMP, ADP and ATP. However, the ^{14}C activity in these nucleotides may actually have been due to a "tailing off" of the large amount of ^{14}C-adenosine added to the column, as no definitive ^{14}C-labeled peaks were identifiable in the areas containing carrier AMP, ADP and ATP.

6. Effect of pH on ^{14}C-ATP Breakdown in Whole Blood

The effect of pH on ^{14}C-ATP breakdown in whole blood was studied by continuously gassing blood samples with gas mixtures containing varied amounts of carbon dioxide (5, 10, 20 or 30% carbon dioxide in air). The gas mixture was saturated with water by bubbling through distilled water to prevent drying of the blood.

To compare ^{14}C-ATP breakdown in high versus low pH whole blood, in each experiment one 2 ml whole blood sample was

continuously gassed with CO_2, while a second 2 ml sample from the same animal was exposed continuously to room air. Two other blood samples from the same animal were simultaneously treated in the same manner for pH determination. For quantitative determination of ^{14}C-ATP degradation the samples were treated as previously described in the section on breakdown of ^{14}C-ATP in whole blood.

7. IMP Separation

The chromatographic separation previously described was not able to separate individual nucleosides or AMP from IMP. Since it is controversial as to whether AMP is dephosphorylated or deaminated in blood, a separation similar to that of Hurlbert et al. (1954) was utilized to separate AMP and IMP and is shown in the lower portion of Figure 3.

Dowex 1 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. One-half ml of a carrier nucleotide solution containing ATP, ADP, AMP, IMP and adenosine was added to a 2 ml blood sample gassed with 5% CO_2, balance air, and incubated for 1 1/2 minutes with a 50 λ sample of ^{14}C-ATP. After treatment with perchloric acid and titration with potassium carbonate, the sample was added to the column for chromatographic separation. At approximately tube 90 the

elution solution was changed to 1.0 M ammonium formate to elute ADP. The elution solution was changed to 1.75 M ammonium formate at approximately tube 120 to elute ATP. Optical density readings were measured at 260 mμ. Peak readings were re-read at 248 mμ, the wavelength of maximum molar absorbancy of IMP, to identify the IMP peak. The results of a separation of carrier nucleotides using this system are shown in the lower diagram of Figure 3.

8. EDTA Studies

The effect of EDTA on the formed elements of blood was studied to determine if EDTA could cause release of intracellular ATP. A 5 ml sample of blood was incubated with 50 μCi of ^{14}C-adenosine (0.24 mg) in 1 ml of saline for 20 minutes at 37.5° C with gentle swirling. The tube was siliconized and sealed with parafilm to minimize drying and hemolysis. Three-fourths ml of a 3% EDTA solution (experimental) or 0.75 ml of isotonic saline (control) was then added to the blood and gently mixed. To allow comparison to the studies of Chen et al. (1972), the sample was then immediately placed in ice and incubated for an additional 5 minutes. The blood was then centrifuged at 42,000 x G for 10 minutes at -3°C. Plasma was carefully removed with a Pasteur pipette and recentrifuged at 42,000 x G for 10 minutes. The isolated formed elements were immediately treated with

7 ml of perchloric acid. The supernatant plasma was removed with a Pasteur pipette after the second centrifugation and 7 ml of perchloric acid was then added to this supernatant. One-half ml of carrier nucleotides were added to both the perchloric acid-treated plasma and to the perchloric acid-treated formed elements. Both samples were then centrifuged at 10,000 x G for 10 minutes at -3°C to remove denatured proteins. Supernatants were decanted, titrated with 5 M potassium carbonate and immediately added to columns for nucleotide separation by ion exchange chromatography.

To determine if pH caused the release of adenine nucleotides from the formed elements of the blood, a 5 ml whole blood sample treated in the same manner as described above, except for omitting the addition of saline or EDTA, was gassed with 5% carbon dioxide in air during the incubation period.

9. In Vivo Studies

Four mongrel dogs of both sexes weighing between 7 and 14 kg (mean = 10.5 kg) were obtained from C.L.A.R. and anesthetized initially with 25 mg sodium pentobarbital per kg body weight intravenously. A sustaining dosage of 6 mg/kg body weight was administered intravenously when a strong corneal reflex was observed.

(a) Plasma ^{14}C-ATP uptake and breakdown by the lungs

The external jugular vein was cannulated proximally with polyethylene tubing (PE 160) for injection into the circulation to the lungs. The tip of the cannula was threaded to the right atrium. The left carotid artery was cannulated with PE 320 proximally with the cannula tip inserted to the aortic arch for collection of blood from the lungs. An injection solution was prepared by mixing 1 ml ^{14}C-ATP (20 µCi; 23 µg ATP) and 2 ml of an isotonic sucrose solution. The sucrose was used as an indicator of dilution of the injection solution in the extracellular fluid volume (Mulrow et al., 1956). Seven-hundredths ml of the injection solution was added to 5 ml of distilled water for sucrose and ^{14}C analysis. The remaining 2.9 ml of the injection solution was injected via the external jugular cannula into the right atrium. Three seconds after injection, blood was collected from the aortic arch directly into 25 ml of 6% perchloric acid in a 50 ml graduated cylinder; collection was for approximately 5 seconds, until the 50 ml mark of the graduated cylinder was reached. One-half ml of carrier nucleotides were added to the sample, and then it was mixed and centrifuged at 10,000 x G for 10 minutes at -3°C. The protein-free filtrate was decanted and titrated with 5 M potassium carbonate. The protein-free supernatant was then added to a resin column for nucleotide separation. A small (1 ml) sample of the protein free filtrate was used for ^{14}C-recovery and sucrose

determination. Injection solution and arterial whole blood sucrose analyses were performed simultaneously using the method of Walser et al. (1955). Per cent ^{14}C by-passing the lungs was calculated by dividing the per cent of the injected ^{14}C recovered in the blood collected by the per cent sucrose recovered in the blood collected, times 100. Subtraction of the per cent ^{14}C by-passing the lungs from 100 gave the estimated per cent ^{14}C taken up by the lungs.

(b) The effect of ATP injection and continuous infusion on systemic arterial blood pressure

Arterial blood pressure was measured from a PE 90 cannula in the femoral artery using a pressure transducer and a Grass polygraph. The injection and infusion sites for ATP were the aortic arch, femoral vein and right atrium, which were cannulated with PE tubing inserted into the dog via the carotid artery, lateral saphenous vein and external jugular vein, respectively.

The solution for single injection and continuous infusion of ATP was prepared by adding 100 mg of ATP to 100 ml of isotonic saline. This solution was injected in quantities ranging from 0.1 ml to 2 ml and was infused at rates ranging from 1 ml/min. to 8 ml/min., depending on the amount required to give an easily recordable change in arterial blood pressure.

10. Reagents

1. ^{14}C-ATP.--Uniformly labeled ^{14}C-ATP was obtained from New England Nuclear (Lot #651-162; 0.345 mg ATP/15 ml 50% ethanol; specific activity 520.9 mCi/mM; and Lot #767-052; 0.43 mg ATP/15 ml 50% ethanol; specific activity 405.4 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.

2. ^{14}C-Adenosine.--Adenosine-8-^{14}C was obtained from Cal Atomic (Lot #000-981; 50 µCi; specific activity 53.5 mCi/mM). The shipment of ^{14}C-adenosine arrived as a dried powder and was maintained as such at -15°C until use. Just prior to use 1 ml of isotonic saline was added to a 50 µCi sample (one vial) for convenience when adding the isotope to the blood.

3. Carrier Nucleotides.--Adenosine and Ba-ADP 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, and 60 mg ATP were mixed with 4 ml distilled water. The solution was heated slightly with hot tap water to dissolve the compounds completely. Twenty-five mg of Ba-ADP were mixed with 4 ml of 0.2 M sodium sulfate. The solution was then centrifuged for 7 minutes. The supernatant was decanted

and added to the nucleotide solution previously prepared, thus producing a solution containing 0.625 mg/ml adenosine, 2.5 mg/ml AMP, 3.1 mg/ml ADP, and 7.5 mg/ml ATP. One-half ml of this solution was added to each sample that was to be fractionated by ion exchange chromatography.

In experiments in which IMP was studied, 35 mg of IMP were dissolved in the solution described above. The IMP was obtained from Sigma Chemical Company.

4. Heparin.--Fifty mg of sodium heparin obtained from Nutritional Biochemical Corporation were added to 1 ml of distilled water. A 20 λ pipette of this solution was added to the blood collection and dried before each experiment. A 1% solution of heparin in saline was prepared. Four ml of this solution were added to 500 ml of saline for preventing coagulation in the polyethylene cannulas used in the in vivo experiments.

5. Ammonium Formate.--A 1.75 M ammonium formate elution solution was prepared by adding 18 liters of distilled water to 1980 g 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.

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

6. 6% Perchloric Acid.--A 6% perchloric acid solution was prepared by adding 7.7 ml of a reagent grade 70% perchloric acid solution obtained from Mallinckrodt Company to a 150 ml graduated cylinder and diluting to 150 ml with distilled water.

7. 2% Sodium citrate.--A solution of sodium citrate was prepared by adding 3.16 g of sodium citrate to a volumetric flask and filling to the 100 ml level with distilled water. The osmolality of the resulting solution was measured on an Osmette osmometer and was approximately 289 mOsm. A 0.22 ml sample of this solution was added to 2 ml of whole blood in order to determine the effect of citrate upon ^{14}C-ATP breakdown.

8. 3% EDTA.--A 10% EDTA solution was prepared by adding 100 ml of distilled water to 10 g of diNa-EDTA. A 33.3 ml sample of the 10% solution was added to a 100 ml graduated cylinder and diluted to the 100 ml level with isotonic saline. Three-fourths ml of the 3% solution was added to 5 ml of whole blood for determining the effect of EDTA on ^{14}C-ATP breakdown in whole blood.

9. Sucrose.--A 300 mosm/l isotonic sucrose solution was prepared by adding 1.03 g of sucrose to 10 ml of distilled water. Approximately 2 ml of this solution along with 1 ml of ^{14}C-ATP were injected into the blood going to the lungs.

10. 4 N Formic acid.--A 4 N formic acid elution solution for gradient elution ion exchange chromatography was prepared by diluting 209 g of 88% formic acid to 1 liter with distilled water.

RESULTS

1. Breakdown of ^{14}C-ATP in Plasma

^{14}C-ATP was added to heparinized fresh dog platelet-free plasma and incubated at 37°C for accurately measured intervals of time. The breakdown of ^{14}C-ATP was determined by measuring the plasma ^{14}C activity in nucleosides, AMP, ADP and ATP after the separation of these compounds by ion exchange chromatography (Figure 5). The per cent of the total ^{14}C activity in each of the individual adenine compound peaks was calculated from the raw data shown in Figure 5, and these per cents of total activity plotted against time of incubation are shown in Figure 6. It is evident from both of these figures that ^{14}C-ATP is rapidly broken down in dog plasma, and the metabolic degradation appears to proceed via ADP, AMP and nucleosides. It is also evident that the ^{14}C-ATP is completely destroyed after twenty minutes of incubation; and no evidence of reformation of ^{14}C-ATP was observed as was the case with whole blood described in the following Section 2. The halftime for ^{14}C-ATP breakdown in plasma was estimated to be approximately three minutes. Since the breakdown of ^{14}C-ATP was difficult to measure accurately in incubation periods of less than one minute, this halftime can only be an estimate and may indeed consist of reactions having

Figure 5. ^{14}C-ATP breakdown in isolated plasma incubated in vitro. One μCi of ^{14}C-ATP was added to 2 ml of plasma and incubated for accurately measured durations of time at 37°C. The plasma pH was approximately 7.8 in each experiment. One ml of each fraction collector sample was plated, dried and counted for one minute. ^{14}C-activity peaks were identified by comparison with carrier nucleotide peaks. Each point represents one observation.

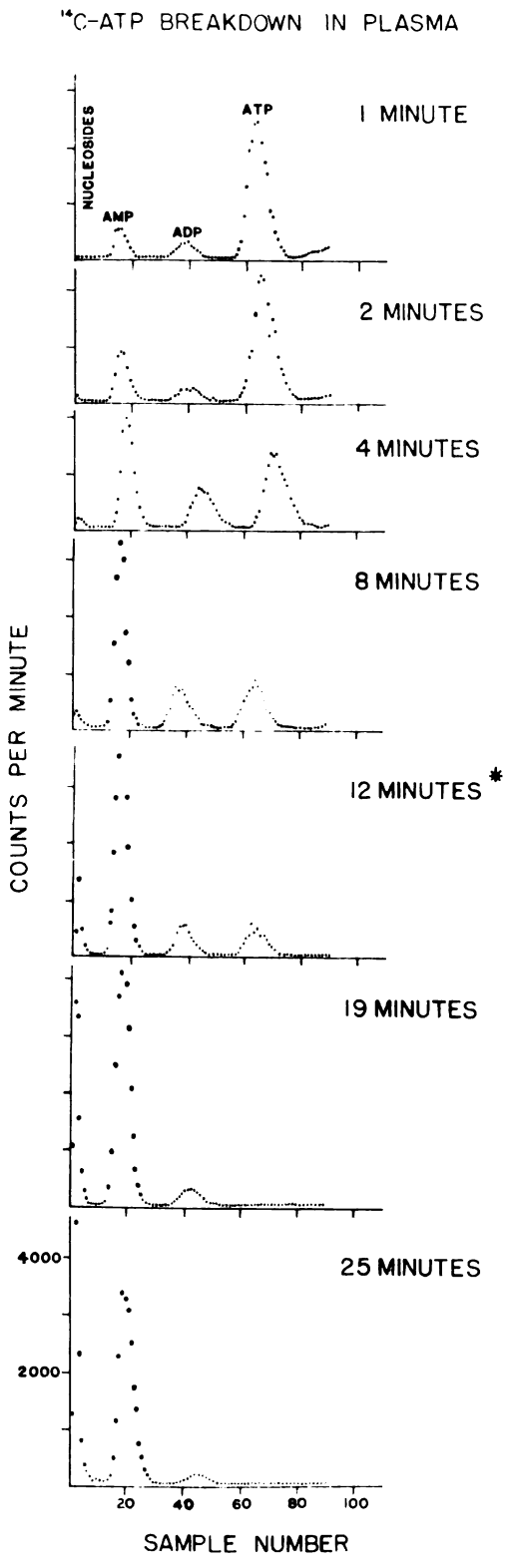
^{14}C-ATP BREAKDOWN IN PLASMA
NUCLEOBIDES
AMP
ADP
ATP
1 MINUTE
2 MINUTES
4 MINUTES
8 MINUTES
12 MINUTES *
19 MINUTES
25 MINUTES
COUNTS PER MINUTE
4000
2000
20
40
60
80
100
SAMPLE NUMBER

Figure 5

Figure 6. Breakdown of ^{14}C-ATP in isolated plasma incubated in vitro. This figure is derived from the experiments in the previous figure. Each point represents one observation and was obtained by dividing total counts/min. in the specified carrier peak area by the total counts eluted from the resin column.

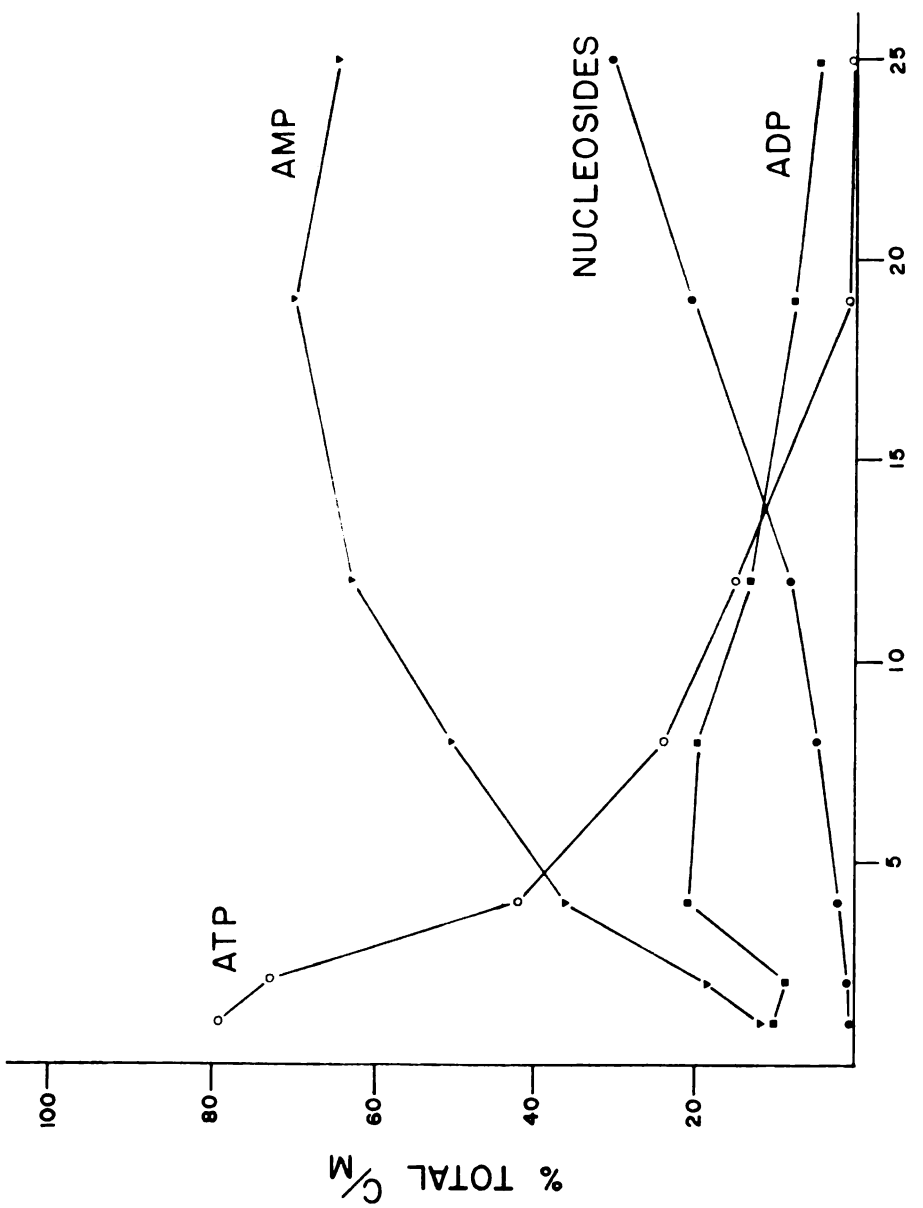
BREAKDOWN OF ^{14}C-ATP IN PLASMA
% TOTAL C/M
100
80
60
40
20
ATP
AMP
NUCLEOSIDES
ADP
5
10
15
20
25
MINUTES

Figure 6

halftimes considerably faster than the one estimated by our crude techniques. The results of these experiments do indicate, however, that ATP released by an organ into the circulating plasma would not be broken down by the plasma to a significant degree in the few seconds during transit through an organ. These studies do not rule out the possibility that platelet, erythrocyte and leucocyte membranes contribute significantly to the rate of ATP breakdown in plasma of whole blood.

2. Breakdown of ^{14}C-ATP in Whole Blood

Figure 7 shows the time course of the breakdown of ^{14}C-ATP added to whole blood. It is evident that the ^{14}C-ATP breakdown is clearly faster in whole blood than in the previously described experiments on isolated plasma; and the estimated half-time for ^{14}C-ATP breakdown in whole blood is 1 1/2 minutes. After 5 minutes of incubation in whole blood the ^{14}C-ATP is almost completely broken down. By 7 minutes of incubation in whole blood there is evidence of resynthesis of ^{14}C-ATP, which continues to occur even after 22 minutes incubation time.

The experiments shown in Figure 7 were performed on bloods from several dogs, and it should be noted that considerable variability in the rate of ^{14}C-ATP breakdown was observed. One factor contributing to this variability was

Figure 7. ^{14}C-ATP breakdown in whole blood incubated in vitro. One μCi of ^{14}C-ATP was added to whole blood and incubated at 37°C for accurately measured intervals of time. The pH ranged from 7.5 to 7.8. After incubation the blood was immediately deproteinized and chromatographed. One ml of each fraction collector sample was plated, dried, and counted for one minute. Values were obtained by dividing total C/M in the ATP area by total C/M eluted from the resin column. The point at 1 1/2 minutes is a mean ± SE of 33 experiments. The remaining points are means of from one to six experiments.

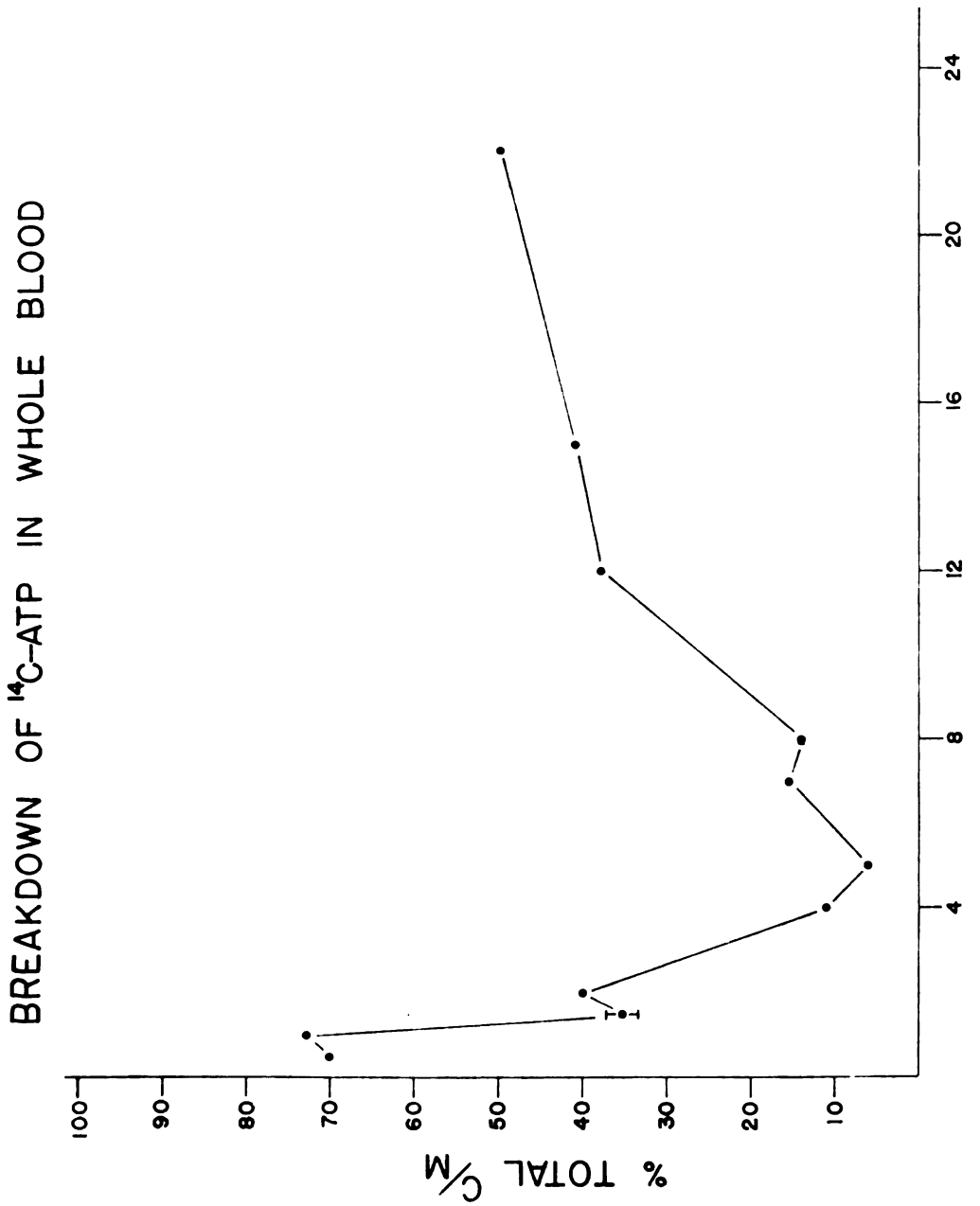
BREAKDOWN OF ^{14}C-ATP IN WHOLE BLOOD
% TOTAL C/M
100
90
80
70
60
50
40
30
20
10
4
8
12
16
20
24
MINUTES

Figure 7

lack of pH control. These experiments were performed with the bloods incubated in air; thus, with different durations of preincubation different CO_2 losses from the blood would occur; hence, the pH varied between 7.5 and 7.7. The hematocrits of the bloods used in the experiments shown in Figure 7 were also measured; however, no correlation between ^{14}C-ATP breakdown rate and hematocrit was observed.

Table 2 shows the breakdown of ^{14}C-ATP and resulting products in whole blood expressed as per cent of the total ^{14}C activity remaining at selected incubation times. ^{14}C-ATP underwent rapid breakdown and then resynthesis. ^{14}C-ADP levels increase slightly and then decline. This observation supports data obtained by Mills (1966), showing that 80 per cent of ATP dephosphorylation in isolated plasma occurs by the direct splitting off of the two terminal phosphates. Also this observation in conjunction with results to be described later (Table 9) seems to indicate a relatively small intracellular ADP pool. ^{14}C-AMP, after an initial increase, is rapidly converted to nucleosides, probably adenosine. ^{14}C-nucleoside levels seem to gradually increase initially and then decline after 15 minutes due to uptake into cells and subsequent resynthesis into ^{14}C-ATP.

It should be noted that the values cited in Table 2 are means. A large variation was noted in the individual observations at the specified times. An example of the degree of

Table 2.--^{14}C-ATP Breakdown at Selected Incubation Times in Whole Blood at 37°C

	Per cent of Initial C/M			
	1 1/2 minutes	4 minutes	15 minutes	22 minutes
ATP	42	20	41	50
ADP	15	20	6	7
AMP	41	26	11	10
Nucleosides	2	32	41	32

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variation at one time of incubation is shown in Table 3. Thus, the data can only indicate qualitative trends in the formation and breakdown of the ^{14}C-adenosine compounds and acceptance of these data as quantitative would be risky.

An experiment was done to determine whether ^{14}C-ATP resynthesis was occurring in the cells, in the plasma, or both. ^{14}C-ATP was incubated in whole blood at 37°C for twenty minutes. The blood was then centrifuged at 0°C at 10,000 X G for ten minutes. Plasma was decanted and 7 ml of perchloric acid was added to the separated formed elements and plasma, and the mixtures were then centrifuged at 10,000 X G for ten minutes to remove coagulated protein. The protein-free supernatants were decanted. Both supernatants were then poured onto columns of Dowex 1 resin for nucleotide separation. Figure 8 shows that ^{14}C-ATP was found only in the cells. Although only a single experiment was done in this manner to establish the intracellular reformation of ^{14}C-ATP and lack of reformation of ^{14}C-ATP in the plasma, a series of experiments with ^{14}C-adenosine confirmed this finding. These latter experiments are described in Section 8 of these results.

Two experiments were done using an elution procedure which would distinguish AMP from IMP. Figure 9 shows the data from one of these two similar experiments and indicates that virtually no IMP is formed during ^{14}C-ATP breakdown in gassed whole blood after 1 1/2 minutes.

Table 3.--Breakdown of ^{14}C-ATP in Whole Blood at 37°C in 1 1/2 minutes with Uncontrolled pH

		Per cent of Initial C/M			
Exp.	pH	ATP	ADP	AMP	Nucleosides
1	7.80	30	24	44	2
2	7.75	38	12	49	1
3	7.60	63	16	19	2
4	7.60	32	12	55	1
5	7.40	34	11	53	2
6	7.80	33	26	39	2
7	7.60	68	11	17	4
8	7.60	58	10	31	1
9	7.60	22	11	65	2
Mean ± SE		42 ± 5.5	14.8 ± 2.0	41.3 ± 5.5	1.9 ± 0.3

Figure 8. Distribution of ^{14}C activity in whole blood after 20 minutes of in vitro incubation with ^{14}C-ATP. One μCi of ^{14}C-ATP was added to 2 ml of whole blood and incubated at 37°C for 20 minutes. The pH was 7.6. The blood was then immediately centrifuged at -3°C to separate plasma and cells. Each fraction was then treated with perchloric acid, neutralized and chromatographically separated. One ml of each fraction collector sample was plated, dried and counted for one minute. Each point represents one observation. The pattern of nucleotide elution is the same as the upper curve in Figure 3.

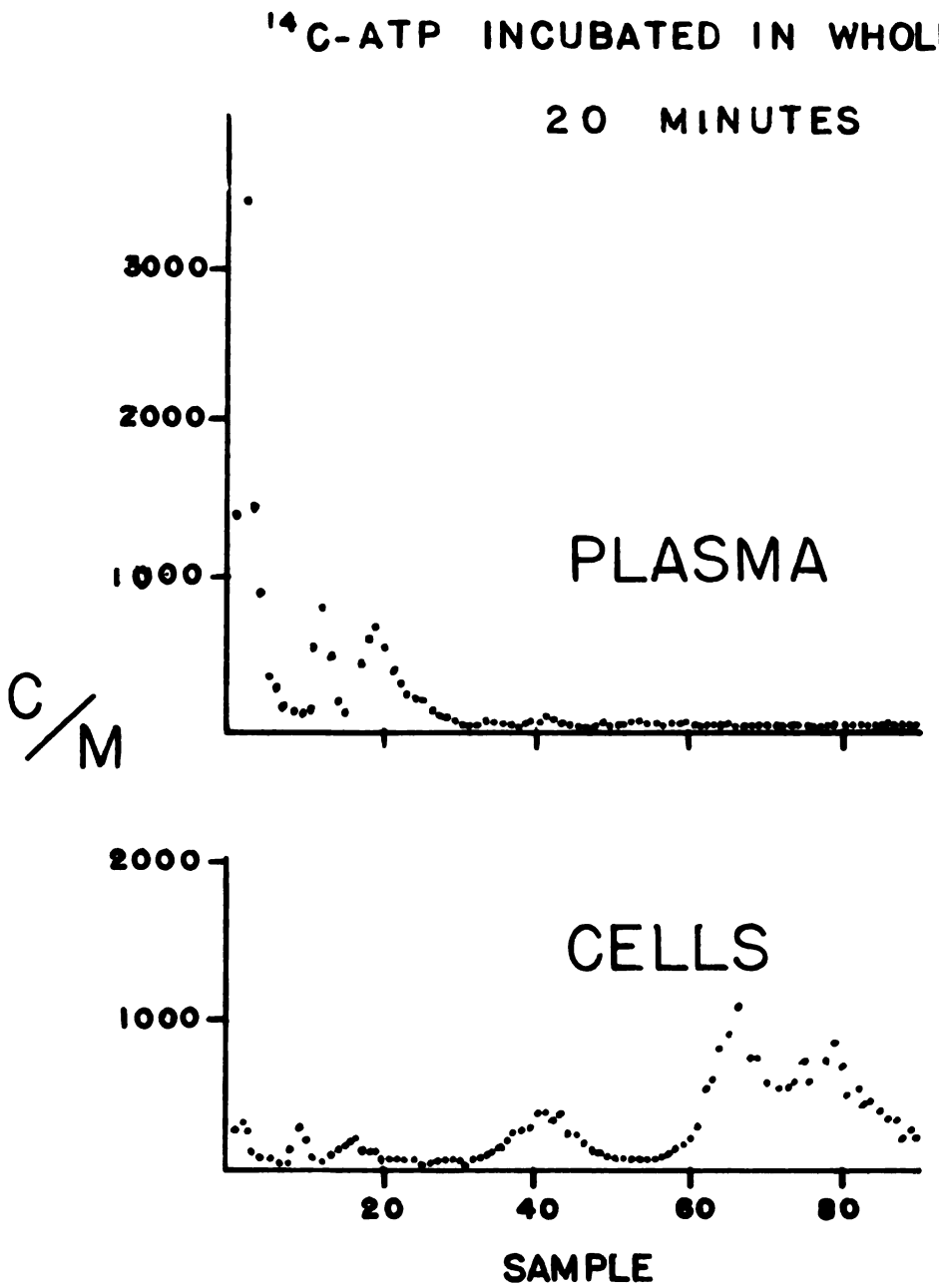
^{14}C-ATP INCUBATED IN WHOLE BLOOD
20 MINUTES
3000
2000
1000
PLASMA
C/M
2000
1000
CELLS
20
40
60
80
SAMPLE

Figure 8

Figure 9. Breakdown of ^{14}C-ATP in whole blood in 1 1/2 minutes at 37°C in vitro. ^{14}C-ATP breakdown in whole blood was studied as described in Figure 7, except that the blood was gassed with 5% CO_2 in air. The pH was 7.34. The nucleotides were separated chromatographically as described in the lower diagram of Figure 3 to separate AMP from IMP. Each point represents one observation.

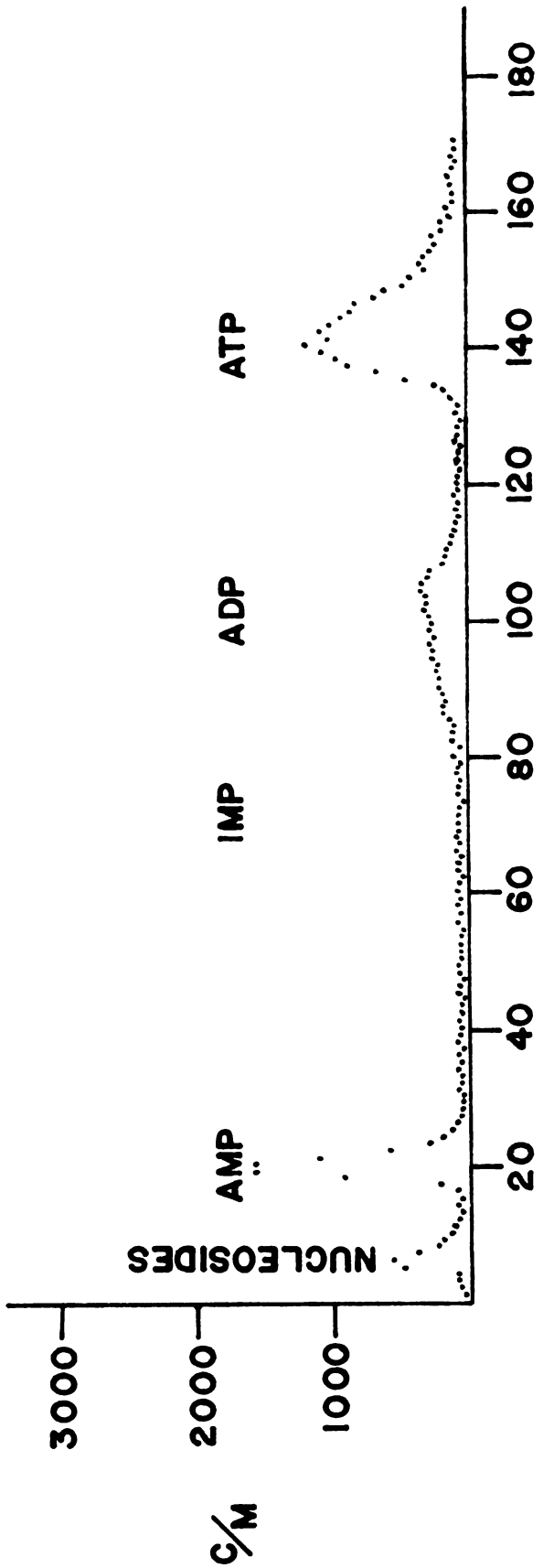
C/M
3000
2000
1000
NUCLEOSIDES
AMP
IMP
ADP
ATP
20
40
60
80
100
120
140
160
180
SAMPLE NUMBER

Figure 9

3. Breakdown of ^{14}C-ATP in Blood with pH Controlled

In order to ascertain if pH changes could account for the large degree of variation in breakdown of ^{14}C-ATP in the experiments shown in Table 3, a series of experiments were performed on whole blood gassed with 5 or 10% CO_2 to control its pH close to 7.40. The per cent ^{14}C-ATP remaining after 1 1/2 minutes of incubation is shown in Table 4. It is evident from these data that considerable variation in ^{14}C-ATP breakdown occurred even though pH was controlled; however, in comparison to experiments without pH control (Table 3), the standard error and range of variation of per cent ^{14}C-ATP remaining after 1 1/2 minutes was considerably less when pH was controlled. Also of possible interest are the observations that: (1) the mean per cent ^{14}C-ATP remaining after 1 1/2 minutes is higher in the gassed (pH controlled) than in the non-gassed bloods; (2) the ^{14}C-ADP is greater in the gassed as compared to the non-gassed bloods; and (3) the ^{14}C-AMP is less in the gassed than in the non-gassed bloods. These findings seem to indicate that increased pCO_2 inhibits ATP and ADP breakdown in the plasma of whole dog blood. However, these experiments also revealed to us that the large variation in ^{14}C-ATP breakdown in either gassed or non-gassed whole bloods from different dogs would make it virtually impossible to study the effect of substances on the rate of ATP breakdown by comparing bloods from different dogs.

Table 4.--^{14}C-ATP Breakdown in Whole Blood at 37°C in 1 1/2 Minutes at pH Approximating 7.40

Experiment	pH	Per cent of Initial C/M			
		ATP	ADP	AMP	Nucleosides
1	7.35	45	22	28	5
2	7.35	35	28	33	3
3	7.38	61	11	27	1
4	7.40	57	21	21	1
5	7.50	52	25	22	1
6	7.42	48	21	29	2
7	7.42	48	21	29	2
8	7.44	49	28	20	3
9	7.45	30	20	49	2
10	7.45	44	29	25	2
11	7.45	61	17	17	5
Mean ± SE		48.2 ± 2.9	22.1 ± 1.6	27.3 ± 2.6	2.5 ± 0.4

4. Studies of ^{14}C-ATP Breakdown in Paired Whole Blood Samples

Table 5 shows the ^{14}C-ATP breakdown rate in two separate samples of blood taken simultaneously from the same dog. Almost identical rates of ^{14}C-ATP breakdown were observed in the individual samples of each pair from the same dog. These observations indicate that variation in ^{14}C-ATP breakdown in whole bloods from different dogs (Table 3) is not caused by inconsistency in the incubation procedure but more likely reflects variation between individual dogs. These observations also made it possible to do reliable comparison studies on blood samples from one animal as described in the following section.

Since heparin influences clotting and since clotting involves the release of nucleotides from platelets, it was suggested that variation in heparin levels could be a possible cause of the variation in ^{14}C-ATP breakdown in whole blood. This supposition was tested experimentally (last 7 minute experiment, Table 5), and it was observed that a 35-fold variation in blood heparin level did not alter ^{14}C-ATP breakdown.

5. Effect of Barium, Citrate and Temperature on ^{14}C-ATP Breakdown in Whole Blood

The inhibitor used to block ATP breakdown in whole blood in previous studies of plasma ATP levels (Chen et al., 1972)

Table 5.--^{14}C-ATP Breakdown in Paired Whole Blood Samples from Individual Dogs

	Per cent of Initial C/M			
Incubation Time	ATP	ADP	AMP	Nucleosides
a) 1 1/2 min.	33	26	39	2
b) 1 1/2 min.	30	24	44	2
a) 1 1/2 min.	26	13	58	3
b) 1 1/2 min.	21	15	61	3
a) 1 1/2 min.	47	19	28	6
b) 1 1/2 min.	44	19	29	8
a) 7 min.	7	7	69	17
b) 7 min.	7	8	70	15
a) 7 min.	20	8	17	55
b) 7 min.	29	8	14	49
a) 7 min.	5	1	72	22
b) 7 min. (35 x Hep)	4	1	73	22

was EDTA. Since the addition of EDTA to whole blood may cause the red cell membrane to become more permeable by chelating calcium, and thereby cause plasma to become contaminated with ATP, another inhibitor of plasma ATP breakdown was sought. The three possible inhibitors of ATP breakdown investigated in this study were: barium (because of its ability to compete with Ca and Mg); citrate (because of its ability to chelate Ca and Mg); and lowered incubation temperature (because of its previous use to block ATP breakdown by Dobson et al., 1971).

Table 6 shows the results of adding 7.8 μMoles of isotonic barium chloride per ml of blood. This should be sufficient barium to overcome physiological calcium and magnesium levels. Sample (a), which had isotonic saline added equal to the volume of the barium solution added, was the control. Barium was added to sample (b). Incubation time was 1 1/2 minutes. Only a slight decrease of doubtful significance in the ^{14}C-ATP breakdown rate was observed with barium treatment. Barium at 7.8 mM is therefore probably not a suitable inhibitor of ATP breakdown.

Forrester (1972) used citrate as an anticoagulant in his later studies on ATP release in active hyperemia. Therefore, the effect of citrate on ATP breakdown was investigated. Table 6 shows the effect of adding 0.22 ml of 2 per cent citrate solution to 2 ml of whole blood. The osmolality of

Table 6.--Effect of Barium, Citrate and Temperature on ^{14}C-ATP Breakdown in Whole Blood in 1 1/2 Minutes

	Per cent of Initial C/M			
Experiment	ATP	ADP	AMP	Nucleosides
a) Saline	63	16	19	2
b) Ba	67	12	19	2
a) Saline	18	12	66	2
b) Citrate	18	27	53	2
a) Saline	49	16	33	2
b) Citrate	56	18	25	1
a) Saline	45	13	38	3
b) Citrate	50	21	27	2
Saline	37.3 ± 9.7*	13.7 ± 1.2**	45.7 ± 10.3	2.3 ± 0.3
Citrate	41.3 ± 11.8	22.0 ± 2.7	35.0 ± 9.0	1.7 ± 0.3
a) 37°	44	23	30	3
b) 3°	79	10	10	2
a) 37°	33	22	43	3
b) 3°	77	9	12	2
a) 37°	37	22	34	8
b) 3°	73	10	14	3
37°	38.0 ± 3.2**	22.3 ± 0.3**	35.7 ± 3.9**	4.7 ± 1.7
3°	76.3 ± 1.7	9.7 ± 0.3	12.0 ± 1.2	2.3 ± 0.3

*Mean ± SE

**Means which are significantly different at the .05 level using the Student's t-test.

the citrate solution was 289 mosm/Kg. The citrate was added to sample (b) in each case shown in the table. For a control, a 0.22 ml sample of isotonic saline was added to sample (a) in each experiment. The pH of the saline and citrate treated-samples did not differ significantly. It can be seen that citrate at the level used in these experiments has no significant effect on the rate of ^{14}C-ATP breakdown, although ^{14}C-ADP was significantly higher in the samples treated with citrate.

Studies by Rosenthal (1948) have shown that temperature is an important factor in the determination of blood pH in vitro; and Scott et al. (1969) have shown that red cell ATP stability in whole blood is pH dependent. Thus, in view of the known biochemical effects of heat, one would expect temperature changes to certainly alter ATP breakdown. Of primary concern, however, is knowledge of the degree to which plasma ATP breakdown is inhibited when blood is cooled as in the studies of Dobson et al. (1971). Table 6 shows the effect of cooling upon the rate of ^{14}C-ATP breakdown in whole blood. Sample (a) in each case was kept at 37°C. Sample (b) was cooled to approximately 3°C. In all three experiments ATP breakdown was much slower in cooled blood than in blood at normal body temperature. Since our stock ^{14}C-ATP was approximately 7% impure, the actual per cent ATP breakdown in 1 1/2 minutes was 55% at 37°C and 18% at 3°C. Of significance

relative to the study of Dobson et al. (1971), it should be noted that considerable breakdown does occur even at 3°C. This rate of ATP degradation at 3°C would leave only about 2% of the initial ATP in 20 minutes. The reduced breakdown of the ^{14}C-ATP at 3°C affects ^{14}C-ADP and ^{14}C-AMP formation more than ^{14}C-nucleoside formation.

6. Effect of Hemolysis on ^{14}C-ATP Breakdown

Chen and Jorgensen (1956) found that ATP breakdown in hemolyzed blood occurred ten times faster than in non-hemolyzed blood. Table 7 shows the effect of hemolysis resulting from chloralose-urethane anesthetic on ^{14}C-ATP breakdown in whole blood gassed with 5 or 10% CO_2. pH ranged from 7.22 to 7.51. By comparison with Table 4, it can be seen that ^{14}C-ATP breakdown is faster in hemolyzed blood. The large variability can in part be explained by different degrees of hemolysis. The effect of chloralose by itself was not studied.

7. Effect of pH on ^{14}C-ATP Breakdown

Jorgensen and Rasmussen (1957) found that addition of an isotonic phosphate buffer or acid-citrate-dextrose (ACD) solution to stored whole blood at 4°C prevented oxypurine formation. They attributed this to a decrease in pH to around 7.1 which resulted from the addition of phosphate buffer or

Table 7.--Effect of Hemolysis on ^{14}C-ATP Breakdown in Gassed Whole Blood in 1 1/2 Minutes (N = 5)

	Mean Per cent of Initial C/M			
Experiment	ATP	ADP	AMP	Nucleosides
Chloralose-Urethane Anesthetic	35.4 ± 6.3*	18.4	38.8	7.2

*Mean ± SE

ACD solution. Scott et al. (1969) found that red cell ATP is pH sensitive in their studies. At pH values below 7.6 to 7.7 it was stable. However, above this range ATP disappeared from the erythrocytes.

Table 8 shows the results of paired whole blood studies designed to test the effect of pH on ^{14}C-ATP breakdown. Sample (a) in each case was gassed with a mixture of carbon dioxide and air; and sample (b) from the same dog was incubated in an unstoppered test tube. Incubation time with ^{14}C-ATP for all samples was 1 1/2 minutes. It can be seen that in almost every experiment the paired blood with the lower pH had less ^{14}C-ATP breakdown. The large amount of variability between experiments may be due to different susceptabilities of different dog bloods to pH change and/or to other conditions prevailing during the incubation times.

Figure 10 shows two types of plots of pH vs. per cent ^{14}C-ATP remaining at 1 1/2 minutes. The experiments used for this figure utilized only dogs anesthetized with sodium pentobarbital, and therefore hemolysis should be minimal. The per cent of ^{14}C-ATP remaining appears to vary inversely with pH; the more alkaline the blood, the greater the amount of ^{14}C-ATP breakdown. Some experiments shown in Figure 10 which were performed at extremely low pH's did not fit the pattern.

Figure 11 shows the effect of pH on ^{14}C-ADP, ^{14}C-AMP and ^{14}C-nucleoside levels. As in the experiments described in

Table 8.--Effect of pH on ^{14}C-ATP Breakdown in Whole Blood in 1 1/2 Minutes

Experiment	pH	Per cent of Initial C/M ATP	ADP	AMP	Nucleosides
1 a)	7.51	20	16	53	11
b)	7.60	14	18	52	15
2 a)	7.48	55	19	24	2
b)	7.60	40	17	37	6
3 a)	7.45	30	20	49	2
b)	7.57	16	20	60	4
4 a)	7.47	23	21	50	6
b)	7.69	19	21	54	7
5 a)	7.42	48	21	29	2
b)	7.71	40	24	32	3
6 a)	7.40	52	25	22	1
b)	7.50	26	17	54	3
7 a)	7.40	57	21	21	4
b)	7.53	32	16	50	1
8 a)	7.38	61	11	27	5
b)	7.51	40	15	44	1
9 a)	7.35	45	22	28	5
b)	7.52	36	23	34	7
10 a)	7.35	35	28	33	3
b)	7.70	19	30	45	6
11 a)	7.29	24	21	46	9
b)	7.63	19	21	49	12

12 a)	7.26	21	23	50	5
b)	7.67	17	23	50	9
13 a)	7.25	37	22	33	7
b)	7.68	32	26	33	9
14 a)	7.22	41	14	38	6.5
b)	7.49	34	16	40	9.5
15 a)	7.17	33	28	35	4
b)	7.63	29	26	38	7
16 a)	7.13	50	14	33	3
b)	7.65	44	18	34	4
17 a)	7.00	48	30	18	4
b)	7.50	36	34	24	6
18 a)	6.94	25	28	45	2
b)	7.64	25	26	46	3
19 a)	6.83	38	32	27	3
b)	7.56	35	33	29	4
20 a)	6.78	56	33	11	0.2
b)	7.71	54	25	20	1
21 a)	7.45	44	29	25	2
b)	7.66	38	27	33	2
22 a)	7.45	61	17	17	5
b)	7.73	55	18	20	7
23 a)	7.28	50	21	26	3
b)	7.52	46	18	33	3
24 a)	7.46	32	22	43	3
b)	7.79	28	27	43	2
25 a)	7.44	49	28	20	3
b)	7.73	39	22	33	6

Figure 10. Effect of pH on ^{14}C-ATP breakdown in whole blood incubated in vitro at 37°C for 1 1/2 minutes. One μCi of ^{14}C-ATP was added to 2 ml of whole blood which was continuously gassed with 5, 10, 20 or 30% CO_2. A second 2 ml blood sample from the same dog was incubated with 1 μCi of ^{14}C-ATP in an unstoppered tube. pH of the gassed samples ranged from 6.78 to 7.47; pH of the non-gassed samples ranged from 7.50 to 7.79. At the end of the incubation each sample was treated with perchloric acid, neutralized, and chromatographically separated. One ml of each sample was plated, dried and counted for 1 minute. Each point in the upper diagram represents one observation and was obtained by dividing the total counts in the ^{14}C-ATP peak by the total counts eluted from the resin column. An x indicates an experiment which may not have been valid since unusually high ^{14}C-ATP levels were found in the non-gassed as well as the gassed sample. Each point in the lower diagram was obtained by plotting the difference in the per cent ^{14}C-ATP against the difference in pH between paired gassed and non-gassed samples for the same experiment shown in the upper diagram. Each point represents one observation.

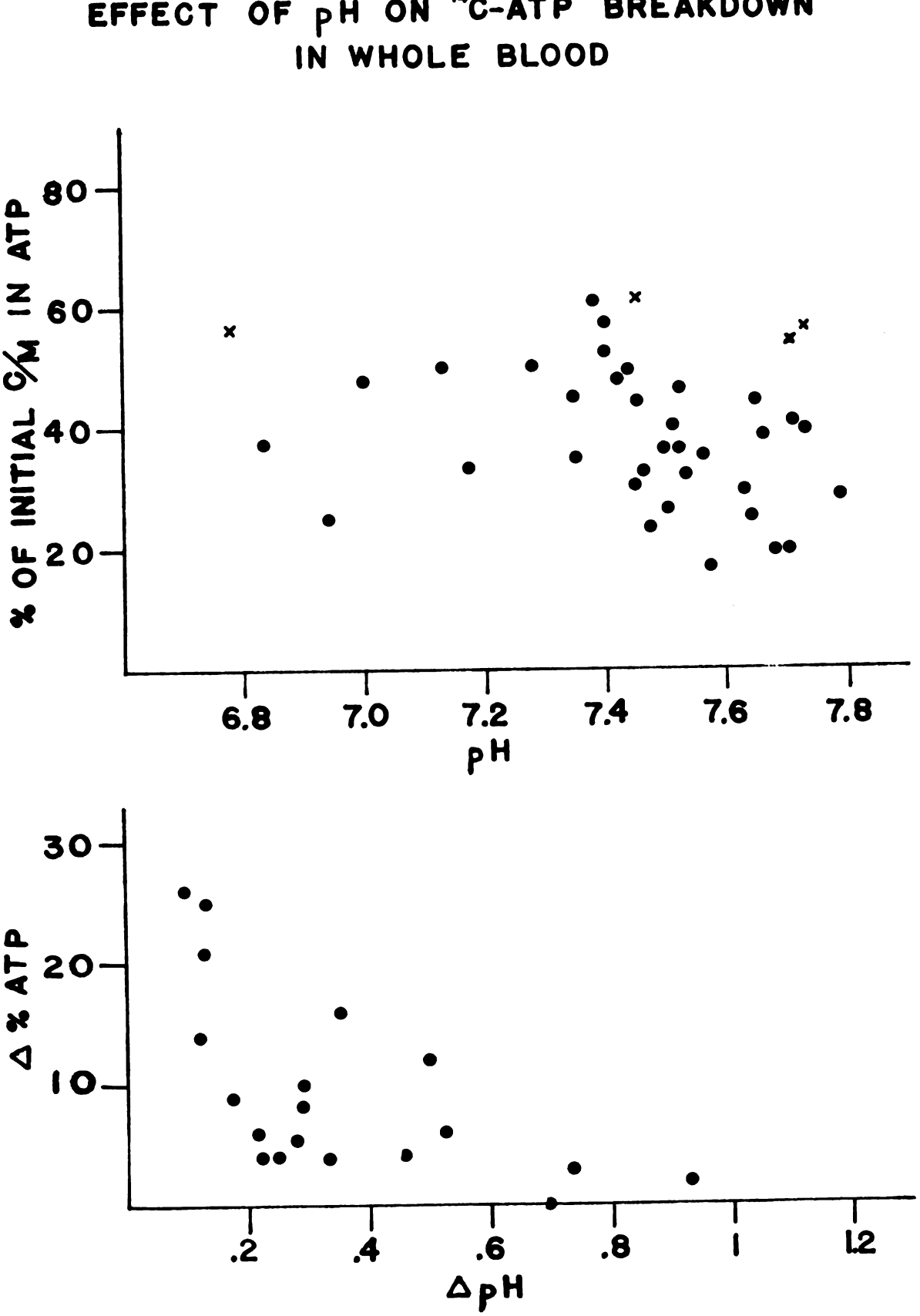
EFFECT OF pH ON ^{14}C-ATP BREAKDOWN IN WHOLE BLOOD
% OF INITIAL C/M IN ATP
80
60
40
20
6.8
7.0
7.2
7.4
7.6
7.8
pH
Δ % ATP
30
20
10
.2
.4
.6
.8
1
1.2
ΔpH

Figure 10

Figure 11. Effect of pH on ^{14}C-ADP, ^{14}C-AMP, and ^{14}C-nucleoside formation in whole blood incubated in vitro with ^{14}C-ATP at 37°C for 1 1/2 minutes. The data in these figures was obtained from the same experiments described in Figure 10. The per cent of total counts/min. for each adenine compound was calculated in the same manner as ATP in Figure 10. Each point represents one observation.

EFFECT OF pH ON ADP, AMP AND NUCLEOSIDE FORMATION FROM ^{14}C-ATP IN WHOLE BLOOD

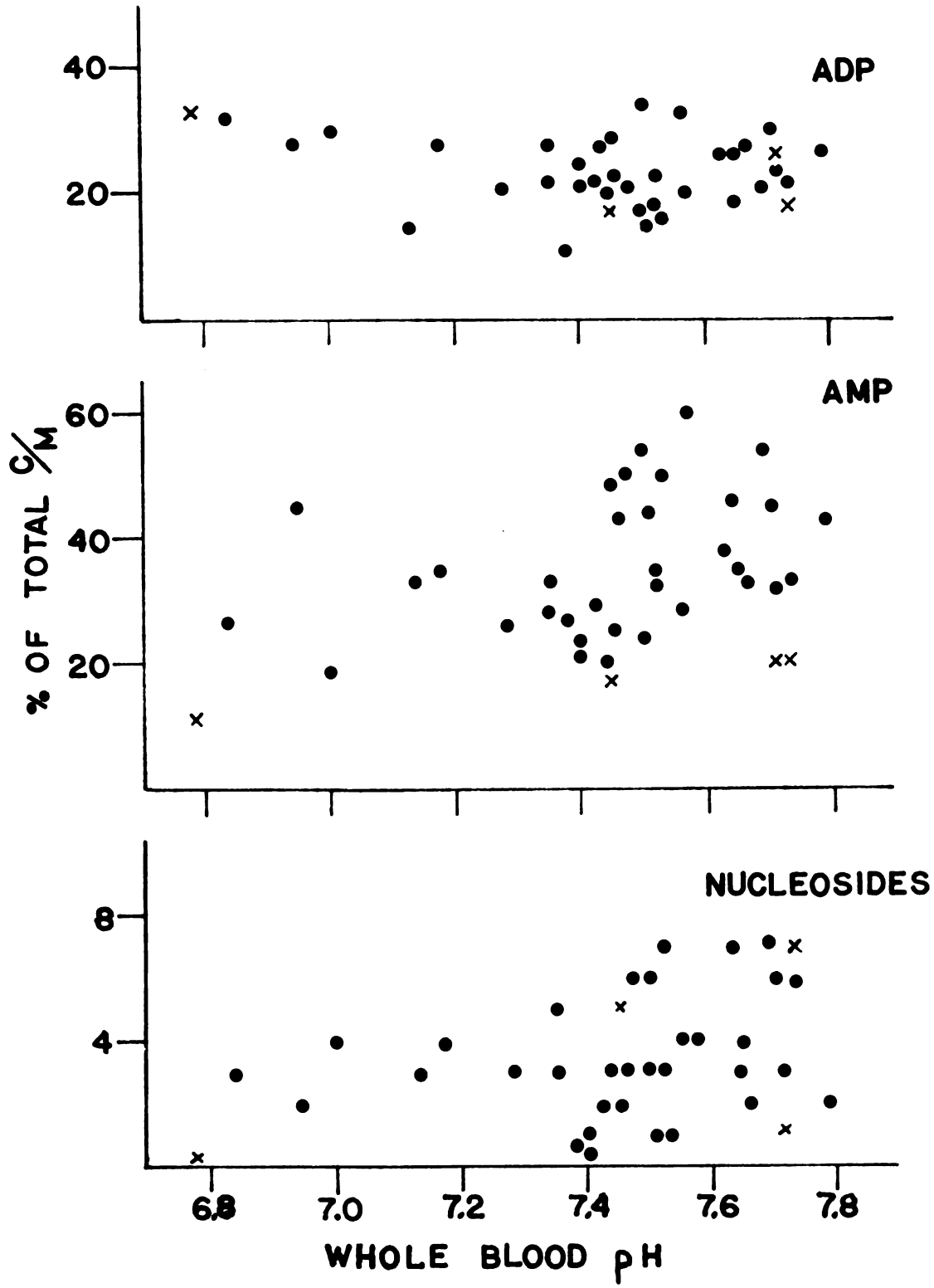

Figure 11

Tables 3 and 4, ^{14}C-AMP levels appear to vary directly with pH; however, in this series of experiments, unlike those in Tables 3 and 4, pH does not appear to affect ^{14}C-ADP or ^{14}C-nucleoside levels.

8. Effect of EDTA on the Formed Elements of Blood

Studies were done to determine if EDTA can cause release of ATP from the formed elements of blood. Intracellular ATP was labeled with ^{14}C by adding 50 μCi of ^{14}C-adenosine to 5 ml whole blood and incubating for 20 minutes; 0.75 ml of EDTA solution or isotonic saline was then added to the labeled blood. There is no consistent difference in plasma ^{14}C-ATP levels between bloods treated with EDTA or isotonic saline in Table 9. In one experiment the blood was gassed with 5% CO_2 and neither saline nor EDTA was added; the plasma ^{14}C-ATP level again did not significantly differ from either saline- or EDTA-treated bloods. Due to the quantitative similarity of the ^{14}C activity in the ATP area to background counting levels and the chromatographic tailing effect of the large amount of ^{14}C-adenosine added, it is probable that this method would not be able to detect the very small differences in plasma ^{14}C-ATP levels which we would suspect to be due to EDTA. In one of the EDTA experiments, however, a ^{14}C peak was clearly defined in the ATP area of the chromatogram. Such a well-defined plasma ^{14}C-ATP peak did not occur

Table 9.--The Effect of EDTA on Plasma ATP Levels

Experiment	Counts Per Minute					
	ATP	ADP	AMP	IMP	Nucleosides	Total
Saline Control						
Plasma	1,300	3,000*	+	---	418,000	437,000
Cells	152,600	35,200	12,300	---	156,000	356,000
Plasma	1,100	2,300	12,800	---	354,000	371,000
Cells	114,000	33,700	10,500	---	124,000	282,000
Gassed Control						
Plasma	500	1,100	26,700	791*	595,000	625,000
Cells	66,000	22,000	14,000	500*	274,000	377,000
EDTA Treated						
Plasma	566	835*	-	---	444,000	449,000
Cells	129,000	14,700	2,900	---	117,000	263,000
Plasma	1,200	2,100*	+	---	360,000	379,000
Cells	126,000	26,000	9,000	---	211,000	372,000
Plasma	1,500	3,200	16,800	---	560,000	582,000
Cells	179,000	28,700	6,000	---	66,900	280,000
Plasma	1,400	853	31,500	974*	878,000	913,000
Cells	122,400	18,600	5,500	---	158,000	305,000

(-) indicates no definitive peak observed.
(+) indicates a definite peak present but not quantifiable due to overlap of adenosine counts.
(*) counts were present in the carrier peak area, but no definite peak could be observed.

in any of the other experiments, although counts above background were present in the ATP area in all experiments. The per cent of intracellular ATP ^{14}C activity found in the plasma was 0.76 in the gassed control, a mean of 0.92 in the saline controls, and a mean of 0.85 in the EDTA-treated samples. Only 0.1 per cent of the intracellular ATP, if found in the plasma, would account for the plasma ATP levels found by Chen et al. (1972) and Forrester (1969). Therefore, the normal existence of ATP in arterial plasma cannot be ruled out by these studies. Likewise, this group of experiments was not able to determine if the ^{14}C activity in the chromatographic ATP area of the EDTA-treated blood was due to an effect of EDTA on the membrane causing outflux of intracellular ^{14}C-ATP.

In these studies considerable ^{14}C-AMP in arterial plasma was observed in six of the seven experiments performed. In the one experiment which did not show ^{14}C-AMP in the plasma, intracellular ^{14}C-AMP levels were also very low. The large plasma ^{14}C-AMP levels apparently indicate AMP formation from adenosine kinase on the surface of the formed elements rather than transmembrane outflux of intracellular ^{14}C-AMP, as intracellular ^{14}C-AMP levels were considerably lower than plasma ^{14}C-AMP levels. This finding also supports the relative stability of AMP compared to ATP in plasma noted in Results, Section 1 (Figure 5), as considerable plasma ^{14}C-AMP remained even after 20 minutes of centrifugation.

In two experiments a chromatographic procedure was used which separated AMP from IMP. Table 9 shows that virtually no ^{14}C-IMP is formed from ^{14}C-adenosine during this incubation time, either intra- or extracellularly. Although ^{14}C activity is present in the IMP area, it is probably due to an adenosine tailing effect, since no definable ^{14}C-IMP peak was observed.

Another fact evident in Table 9 is that plasma ^{14}C-nucleoside levels are in every case considerably higher than those observed intracellularly, suggesting that over the time course of these experiments ^{14}C-adenosine had not equilibrated across the cell membrane.

9. In Vivo Studies on ^{14}C-ATP Breakdown in the Pulmonary Circuit

Folkow (1949) observed that intravenously injected ATP appeared to be rapidly destroyed (inactivated) in the pulmonary circuit. Gordon (1961) obtained results which also indirectly indicated a trapping or rapid breakdown of ATP in the lungs. Figure 12 shows the results of one of four similar experiments in which ^{14}C-ATP and sucrose were injected into the thoracic vena cava and outflow was collected from the aortic arch approximately 5 seconds later. It is apparent that very little ^{14}C-ATP passed out of the lungs and through the left side of the heart. By comparison with sucrose recovery in the aortic arch blood, 83% $\pm$ 3% (mean $\pm$ SE, N = 4)

Figure 12. ^{14}C-ATP breakdown during one passage through the vasculature of the lungs. Twenty μCi of ^{14}C-ATP in 2 ml of isotonic sucrose were injected into the right atrium. Blood was collected 3 seconds later from the aortic arch into perchloric acid. After neutralization and chromatographic separation, 1 ml of each fraction collector sample was plated, dried and counted for one minute. The upper diagram shows a ^{14}C-ATP purity check, performed as described in Figure 4. The lower diagram shows the ^{14}C activity profile after passage of ^{14}C-ATP through the lungs. Each point represents one observation.

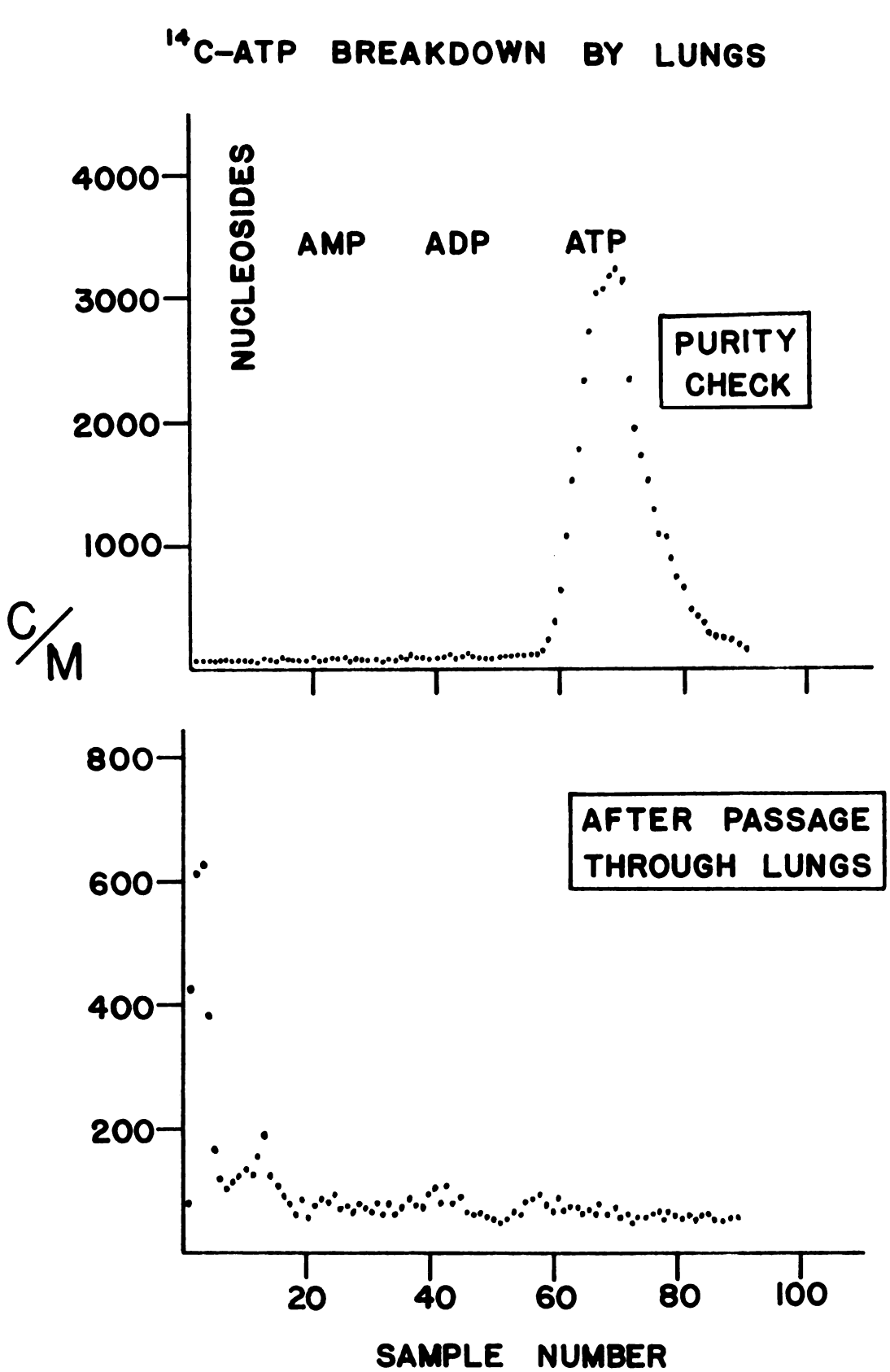
^{14}C-ATP BREAKDOWN BY LUNGS
NUCLEOSIDES
AMP
ADP
ATP
PURITY CHECK
4000
3000
2000
1000
C/M
800
600
400
200
AFTER PASSAGE THROUGH LUNGS
20
40
60
80
100
SAMPLE NUMBER

Figure 12

of the ^{14}C injected was estimated to be taken up by the lungs. The lungs therefore avidly take up ATP from the blood or rapidly convert it to other substances which do not immediately leave the lungs. Of the ^{14}C activity recovered in the blood after the passage of the ^{14}C-ATP injection through the lungs only 17% ± 11% (mean ± SE, N = 4) was still ^{14}C-ATP. The other labeled ^{14}C-ATP degradation products in the blood leaving the lungs were nucleosides, 51% ± 14%; AMP (and/or IMP), 24% ± 6%; and ADP, 7% ± 3% (mean ± SE, N = 4). The breakdown of plasma ATP during passage through the lungs is extremely rapid, as ^{14}C-nucleosides can be recovered from injected ^{14}C-ATP in just six seconds.

10. In Vivo Studies on the Effect of ATP on Systemic Arterial Pressure

Figure 13 shows the results of ATP injection (A) and infusion (B & C) on systemic arterial blood pressure. The results shown are typical of those observed in four experiments performed on different dogs. Injection of 500 µg of ATP into the aortic arch (Figure 13,A) produced a large, transient drop in systemic pressure, whereas 500 µg of ATP injected into the vena cava adjacent to the right atrium resulted in little if any fall in pressure. Infusion of 2 mg ATP/min into the aortic arch (Figure 13,B) produced a large and continuous drop in systemic pressure. Switching the 2 mg/min infusion of ATP into the vena cava at the right atrium, however,

Figure 13. Effect of intravenous and intra-arterial ATP infusion on systemic arterial blood pressure. (A) Five hundred µg ATP were injected in 1 ml saline into the aortic arch followed by 1 ml isotonic saline injected into the same site and then 500 µg ATP injection into the vena cava adjacent to the right atrium. (B) Two mg/min ATP was infused first into the aortic arch and then into the vena cava adjacent to the right atrium. (C) Fifteen mg/min ATP was infused first into the vena cava adjacent to the right atrium, then into the femoral vein, and then back into the right atrium again. Chart speed = .5 cm/20 sec.

EFFECT OF INTRAVENOUS AND INTRAARTERIAL ATP INFUSION ON SYSTEMIC ARTERIAL BLOOD PRESSURE

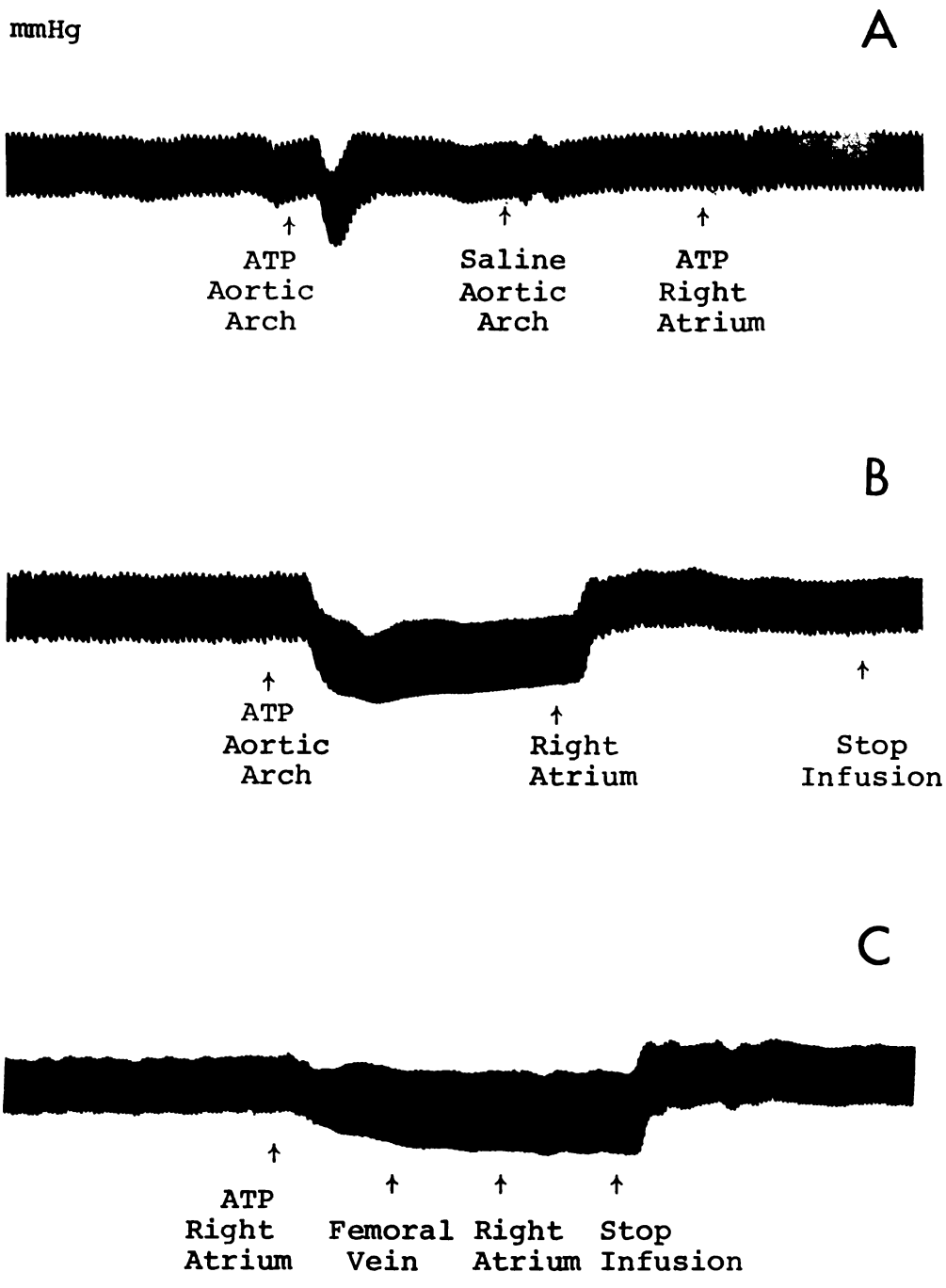

Figure 13

resulted in a return of systemic pressure to control levels; and no further increase in systemic pressure was recorded when the infusion was stopped. It can also be seen that infusion of 15 mg ATP/min into the thoracic vena cava at the right atrium (Figure 13,C) did produce a significant drop in systemic pressure. Switching the infusion of this amount of ATP to the femoral vein did not change the pressure drop. An increase in pressure was recorded when this infusion was stopped. These findings support the uptake and inactivation of ATP by the lungs observed by others (Folkow, 1949, and Gordon, 1961). The slower breakdown of ATP in blood as compared to the lungs is also supported by the lack of changes in systemic blood pressure when a large amount of ATP being infused into the thoracic vena cava is switched into the femoral vein.

Also of interest are the changes in ventilation observed during ATP infusion. An increased ventilation of 31 breaths/min was observed during intravenous infusion of 15 mg ATP/min as compared to a control ventilation rate of 20 breaths/min. Also, increased ventilation was observed when 500 μg of ATP were injected into the aortic arch.

DISCUSSION

The results of in vitro studies on ^{14}C-ATP metabolism in whole blood and plasma showed that added ATP was rapidly broken down. Jorgensen (1956) and Forrester (1972) also found ATP degradation in whole blood and plasma. Jorgensen, however, found a slower rate of ATP breakdown than observed in our studies. In his studies approximately 50% of the added ATP was degraded after two hours in plasma and one-half hour in whole blood compared to three minutes in plasma and 1 1/2 minutes in whole blood reported in the results of this thesis. Furthermore, our studies indicate that ^{14}C-ATP is virtually completely broken down in plasma after 20 minutes, whereas Jorgensen still had measurable ATP after five hours. The slower ATP breakdown observed by Jorgensen may have been due to his use of human rather than dog blood, but more probably was due to: (1) the large quantities of ATP which he used, and (2) his dilution of the blood and plasma with Tyrode-Locke's solution. His ATP concentration added initially to both whole blood and plasma was 400 μmoles/l of diluted plasma or whole blood. The plasma and blood were diluted by adding 1 ml of Tyrode-Locke's solution containing the proper amount of ATP to 4 ml of whole blood. This was

approximately 240 µg of ATP/ml of diluted whole blood or plasma, whereas in our studies only 0.57 µg ATP was added to each ml of undiluted whole blood or plasma. The use of high specific activity ^{14}C-ATP made it possible to add such small quantities of carrier ATP. The 0.57 µg ATP/ml plasma used approached physiological levels observed by Chen et al. (1972) (0.45 µg ATP/ml) in post-tetany femoral venous plasma and Forrester (1972) (0.2 µg ATP/ml) in venous plasma from an exercising human forearm. The dependence of ATP breakdown rate upon initial ATP concentration was shown by Forrester (1972). Approximately one-half of the ATP added to human citrated plasma at 37°C was degraded in 15 minutes when the initial ATP concentration was 10 µg/ml, but in only six minutes at an initial concentration of 1 µg/ml. Thus, the breakdown rate of low levels of ATP in human citrated plasma observed by Forrester was very close to that found in our studies with heparanized dog blood, presumably due to his addition of similar quantities of ATP.

^{14}C-ATP added to whole blood disappeared approximately twice as fast as that added to isolated plasma. This is similar to the findings of Jorgensen (1956), who observed ATP breakdown in whole blood to be 7 to 8 times faster than in isolated plasma. Considerable plasma ATP is evidently broken down by the formed elements in blood, especially the erythrocytes, and an "ecto" ATPase related to thrombosthenin has been

reported for platelets (Chambers et al., 1967, and Mason and Saba, 1969). Even though ATP breakdown in blood is rapid, there is still sufficient time for ATP released by an organ to leave that organ in the venous blood and even to recirculate if ATP breakdown by the lungs could be inhibited.

The resynthesis of ^{14}C-ATP by the formed elements of the blood which occurred in our studies after 7 minutes incubation is in agreement with studies by Parker (1970) and Schrader et al. (1972), although Schrader's studies were done on red cell ghosts. Added ^{14}C-ATP was apparently degraded to adenosine in the plasma via ADP and AMP, as described by Chen and Jorgensen (1957), who used enzymic assays to quantify these nucleotides. The adenosine was then rapidly taken up by the erythrocytes and platelets and rephosphorylated to ATP. Inosine and hypoxanthine may also have been formed, but their specific formation was not detected since the chromatographic separation used in these studies was unable to distinguish between inosine, hypoxanthine and adenosine. The rapid uptake of adenosine by the formed elements and its incorporation into intracellular adenine nucleotides was also found when ^{14}C-adenosine was added to blood. This uptake may provide a mechanism of conserving adenine nucleotides rather than degrading them in the plasma to hypoxanthine and then to uric acid in the liver. The resynthesis of ATP by the formed elements supports Schrader's suggestion that phosphorylation

rather than deamination of adenosine occurs at low external adenosine concentrations due to the lower K_m of adenosine kinase.

The rapid rise in ^{14}C-AMP and the consistently smaller rise in ^{14}C-ADP that occurs during ^{14}C-ATP degradation in plasma and whole blood is in agreement with Mills (1966), who found that 80 per cent of added ATP was directly dephosphorylated via α-β-ATPase in plasma to AMP.

Although considerable variability was observed in the ^{14}C-ATP breakdown rate in whole blood from different dogs in the present studies, ^{14}C-ATP breakdown rates in paired whole bloods from the same dog were very similar. Thus, the analysis of paired whole bloods provided a suitable way to study the effect of various in vitro conditions on ATP breakdown. In an effort to facilitate the study of the physiological role of extracellular ATP, several methods of inhibiting ATP breakdown were tried; however, an effective inhibitor which would be harmless to the body and not affect the cell membrane was not found. Heparin had no effect on ATP breakdown. Barium was investigated in our studies as a possible inhibitor of ATP breakdown but also had no effect. Citrate inhibited breakdown only slightly in the concentration used by Forrester (1972) (15 mMoles citrate/l). Holmsen and Stormorken (1964) found that eighteen times as much citrate as magnesium (1 mMole/l) had to be added to blood to cause marked inhibition of ADP breakdown. Since Forrester used a

citrate concentration smaller than this as an anticoagulant, any ATP present in plasma from resting subjects would presumably have had time to breakdown during the approximately 30 minute period between collection and analysis.

Jorgensen and Poulsen (1955) found that temperature was an important factor in oxypurine formation in blood, much faster accumulation occurring at 37°C than at 4°C. Forrester (1969) found rapid ATP decay in diluted plasma at room temperature. The present studies have shown that significant ^{14}C-ATP degradation can occur even at very low temperatures. After 1 1/2 minutes, approximately 20 per cent of added ATP was degraded in whole blood incubated in an ice bath. This finding could explain why Katori and Berne (1966) found adenosine rather than ATP in the perfusate from hypoxic heart muscle and why Dobson et al. (1971) could find no ATP but significant quantities of adenosine in the cooled (0°C) venous effluent from exercising skeletal muscle, since sufficient time elapsed for much ATP breakdown to occur between sampling and ATP assays.

In the present studies the presence of hemolysis seemed to greatly accelerate ATP breakdown. Chen and Jorgensen (1956) also noted this phenomenon. In our preliminary studies non-siliconized glassware caused slight hemolysis; and also, blood taken from dogs anesthetized with chloralose-urethane was noted to be greatly hemolyzed. In both types of

hemolyzed blood, ^{14}C-ATP breakdown was much faster than in non-hemolyzed blood samples. Thus, it is possible to have lower than normal ATP levels in plasma as a result of hemolysis even though ATP is released from the red cells in this circumstance.

In order to estimate the ATP breakdown that occurs in plasma of the intact animal, the breakdown of ^{14}C-ATP in whole blood in vitro was studied. During the course of this study it became evident that there was considerable variability in ATP breakdown rate in bloods from different dogs. Non-gassed blood samples had a wide variation in ATP breakdown rate when incubated for 1 1/2 minutes. Gassing the blood with specific mixtures of CO_2, however, enabled much more consistent results to be obtained. The ^{14}C-ATP added to gassed blood was degraded 51.8 $\pm$ 2.9% (Mean $\pm$ SE, N = 11) in 1 1/2 minutes. This per cent breakdown in 1 1/2 minutes can now be used for comparison with other studies to determine if ATP breakdown in whole blood is significantly altered by pathological conditions such as hypertension.

Bishop (1960) found that gassed blood samples lost ATP far less rapidly than non-gassed samples. This effect was attributed to pH differences between the two samples, the non-gassed sample having a higher pH and therefore losing ATP more rapidly. Jorgensen and Grove-Rasmussen (1957) found that adjustment of pH to 7.1 or lower in stored blood that was drawn through an ion-exchange column prevented oxypurine

accumulation and therefore ATP degradation. Beutler and Duron (1963) found that at 4°C red cell ATP disappeared rapidly at pH 7.5 to 7.9 but was quite stable at low pH (6.8 to 7.2). At 37°C, however, they noted an opposite pH effect; that is, greater stability at high pH. Scott et al. (1969) found that red cell ATP was lost rapidly at pH values above 7.6-7.7 at 25°C. The lost ATP was recovered by lowering the pH. At 37°C ATP was rapidly lost above pH 7.4-7.5. The increased glycolysis which occurs at 37°C could in part explain ATP preservation at this temperature at elevated pH values. Rosenthal (1948) found that the pH of blood in vitro was very dependent upon temperature. The pH rose with a fall in temperature in a linear manner.

The present pH studies were more concerned with effects on ATP in the plasma rather than on red cell ATP per se. Therefore, it is questionable as to whether the conclusions of some of the previous studies described above can be applied here, as they dealt with pH effects on red cell ATP. pH was found to have a definite effect on added ATP breakdown rate. ^{14}C-ATP appeared to be more stable in bloods having pH between 7.2 and 7.4 at 37°C; however, this can only be an approximation due to the small number of samples below pH 7.3. Both above and below this range, breakdown rate seemed to increase. Breakdown was always inhibited in the paired sample with the lower pH down to approximately pH 6.94. Below pH 6.94

breakdown was inhibited only slightly when compared to the sample that was not gassed. ^{14}C-ATP and ^{14}C-AMP appeared to vary inversely with each other after 1 1/2 minutes incubation at different pH values. ^{14}C-ADP and nucleosides were not affected in a consistent manner by pH.

Fleishman et al. (1957) found active small vessel dilation in response to an increase in hydrogen ion concentration in the dog foreleg, the effect occurring predominantly on the alkaline side of pH 7.3. At pH 7.6 vessel resistance was significantly increased. A similar response to pH change occurred in the kidney (Emanuel et al., 1957). The mechanism of the vessel response was not known. However, since it has been suggested that ATP may exist in arterial plasma and since the results presented in this thesis indicate that ATP breakdown is less in more acid bloods, it is possible that ATP may be the mediator of the pH effect. Stowe et al. (1973) found that pH decreased to a minimum of 7.1 during active hyperemia in skeletal muscle with constant flow. With natural flow the decrease would probably be less. The present finding that ATP is most stable in bloods with decreased pH at body temperature also indicates that during active hyperemia ATP released into the blood may have a somewhat longer survival time and may thus act as a dilator longer. The relatively greater instability of ATP at high pH could be another reason why Berne and Dobson found no ATP in their studies, as their

samples were collected in unstoppered tubes and thus would have high pH because of CO_2 loss.

Forrester (1969) and Chen (1972) found approximately 200 ng/ml of ATP in normal arterial human and dog plasma respectively. Forrester (1972), however, attributed his ATP levels to platelet damage and the effect of EDTA on the erythrocyte membrane. Although EDTA is both an anticoagulant and an inhibitor of ATP breakdown, Forrester used EDTA in his early studies as an anticoagulant. After becoming aware of evidence that EDTA increased the permeability of skeletal muscle membranes to ATP (Abood et al., 1962) and after noting that EDTA increases plasma ATP, Forrester used citrate as his anticoagulant. Citrate, however, has been shown in the present studies and by Forrester (1972) to be ineffective in inhibiting ATP breakdown in the concentration used by Forrester for anticoagulation. Thus, the lack of measurable amounts of arterial plasma ATP in Forrester's later studies (1972) on citrated plasma could be caused by either increased plasma ATP breakdown, lack of release of platelet and/or red cell ATP into the plasma, or both.

Abood et al. (1962) found that the presence of EDTA caused greater release of skeletal muscle ATP than a medium without EDTA. Kuperman et al. (1964) found an increased outflux of ^{3}H-AMP from nerve axons immersed in a solution to which EDTA was added as compared to an EDTA-free medium.

However, Scott et al. (1969) found no loss of red cell ATP in EDTA-treated blood when the pH was kept below 7.6. On the other hand, Scott observed evidence that ATP passes out of the red cell in whole blood with pH over 7.6. High pH, therefore, could be the factor causing ATP release in blood rather than EDTA.

The studies reported in this thesis on EDTA-treated blood are not able to determine whether EDTA or high pH cause the release of small quantities of ATP from the formed elements of the blood, since the technique used was not sensitive enough to detect differences in such low ATP levels. However, the ^{14}C-ATP activity in the plasma did appear to vary directly with the amount of intracellular ^{14}C-ATP formed. This provides some evidence for ATP leakage from the cells in both control and EDTA-treated blood. However, the normal existence of ATP or other adenine compounds in arterial plasma is still questionable. Those investigators who have attempted but have not found ATP in plasma used methods which did not significantly inhibit its breakdown; those who did find it used substances such as EDTA which may have altered the cell membrane and thereby caused a leakage of ATP.

The studies on EDTA-treated and control bloods revealed a significant peak of ^{14}C-AMP (or a closely related isomer) in the plasma in both types of bloods. Therefore, AMP can evidently exist in arterial plasma for a considerable period

of time. This fact may indicate that AMP could be clinically useful as a vasodilator, provided its metabolism in vivo is not extremely fast. In this latter regard, Gordon (1961) has reported that AMP is not inactivated by the lungs. Based on the erythrocyte ghost data of Schrader et al. (1972) and the results from the whole blood experiments reported in this thesis, it seems more likely that any adenosine released from the heart during active hyperemia is either phosphorylated to AMP extracellularly or enters the cell and forms adenine nucleotides, rather than forming extracellular inosine and hypoxanthine. The presence of myokinase in myocardial and other cell membranes also makes the conversion of this extracellularly formed AMP to ADP and ATP a possibility, should either extra- or intracellular ATP be available for this reaction. Thus, even though adenosine may be the only adenine compound passing through the cell membrane, extracellularly formed AMP, ADP or ATP present in the plasma or interstitial fluid could also be possible mediators of vasodilation.

The significance of the IMP formation in blood described by others is questionable, as it was not formed in significant quantities during ^{14}C-ATP breakdown in blood or during nucleotide synthesis from ^{14}C-adenosine. Bishop's theory regarding adenylic deaminase in blood seems to be incorrect, although his bloods were hemolyzed in many cases and also

were incubated considerably longer than those studied in this thesis. On the basis of Bishop's observations, it was expected that the large quantity of ^{14}C-adenosine added would certainly result in some intracellular ^{14}C-IMP formation. On the other hand, since virtually none was found, the special inhibition of adenylic acid deaminase in blood found by Conway and Cooke (1939) may be very true indeed, although intracellular compartmentalization of adenine nucleotide formation and AMP deamination may also explain these discrepancies.

The in vivo lung studies when compared to the in vitro whole blood studies of ^{14}C-ATP breakdown illustrate a great difference between ATP stability in isolated blood and in blood passing through an organ. The experimental evidence described in the results of this thesis indicate conclusively that the lungs rapidly take up and degrade ATP, in agreement with the indirect observations of Folkow (1949) and Gordon (1961). Nucleosides were formed from plasma ATP by the lungs in a few seconds, whereas in isolated blood minutes were required. Although the fate of the ATP taken up by the lungs is unknown, the following comments regarding ATP metabolism by the lung can be made. Fishel et al. (1970) have found that mouse lungs contain very little adenyl cyclase, the enzyme which converts ATP to cyclic AMP; thus, this is an unlikely route for lung ATP degradation. On the other hand,

Fishel et al. observed that lung tissue actively converted ATP to AMP. Since ADP and AMP apparently can pass through the lungs unchanged (Brashear and Ross, 1969, and Gordon, 1961), and since very little ^{14}C-ADP and ^{14}C-AMP were found in the pulmonary outflow in our studies after ^{14}C-ATP injection into the right atrium, the α-β-ATPase and β-γ-ATPase reactions must be almost totally absent on the external cellular surfaces of the lungs. This suggests that ATP per se is taken up by lung tissue rather than its degradation products. Clarke et al. (1952) found high adenosine deaminase levels in lung tissue, and Pfleger (1969) found rapid adenosine uptake by the lungs (90 per cent uptake compared to 10 per cent breakdown). The metabolic fate of the ATP and adenosine taken up from the plasma by the lungs remains to be elucidated.

ATP infusion studies presented in the Results give us indirect and inconclusive evidence that only high levels of ATP can pass through the vasculature of the lungs. ATP infused into the right atrium or femoral vein produced approximately the same amount of vasodilation, suggesting but not proving that little breakdown of ATP occurred in blood travelling from the hindlimb to the lung, in agreement with the reported in vitro blood studies.

The lung studies, in conjunction with the breakdown rate studies in isolated blood, indicate that ATP could recirculate

through the body if it could survive passage through the lungs. Thus, the vasodilator potency of ATP could be utilized in conditions such as hypertension if a suitable inhibitor of ATP breakdown in the lung could be found. Perhaps dipyridamole, which blocks adenosine uptake by the lungs, would also block the uptake of ATP. The fate of ATP in passing through other organs remains to be investigated.

It was suggested in the Literature Review that the mechanism of action of ATP may be similar although not necessarily identical to that of actylcholine, as both compounds depress smooth muscle contraction in many body tissues. Although the actual mechanism by which the smooth muscle relaxation is produced by extracellular ATP is unknown, several distinct possibilities exist. ATP could decrease membrane permeability to calcium, thereby decreasing intracellular free calcium levels and thereby increasing the effectiveness of relaxing protein (troponin, tropomyosin). A second possibility would be hyperpolarization of cells by ATP. This could occur either by an increased activity of the pump extruding sodium from the cell, by decreased membrane permeability to sodium, or by increased membrane permeability to potassium. In the case of a coupled sodium-potassium pump, an increased pump rate would also cause membrane hyperpolarization by increasing the rate of potassium diffusion out of the cell. More evidence is needed to elicidate the actual

mechanism of ATP action and to relate the extracellular nucleotide profile to physiological regulation.

SUMMARY AND CONCLUSIONS

A gradient elution ion exchange chromatographic procedure was utilized to investigate the breakdown of ^{14}C-ATP in dog whole blood and plasma in vitro. Metabolism of ^{14}C-ATP by the lungs was studied in vivo; and ^{14}C-adenosine metabolism was studied in whole blood in vitro. ^{14}C-ATP was degraded at a faster rate in whole blood than in plasma. However, even in whole blood the survival time of the ^{14}C-ATP was sufficiently long to permit recirculation in the body. ^{14}C-ATP breakdown products in both plasma and whole blood were ADP, AMP, and nucleosides. Hemolysis apparently increased ^{14}C-ATP breakdown rate, whereas cold decreased the rate. Citrate, barium, and heparin had little, if any effect on breakdown rate. Decreasing whole blood pH consistently inhibited ^{14}C-ATP breakdown rate except when below pH 6.94. ^{14}C-ATP survival time in plasma of whole blood was greatest at about pH 7.2 to 7.4, indicating that any ATP released into the acidic blood flowing through a limb during exercise hyperemia may have a longer time in which to exert its vasodilatory effects. In vivo studies indicated that ^{14}C-ATP was rapidly removed from the plasma by the pulmonary circuit. Eighty-three per cent of ^{14}C intravenously injected as ^{14}C-ATP was

taken up by the lungs during a single pass. Of the 17 per cent ^{14}C activity recovered after a single pass through the lungs, only 17 per cent was still ATP. It is suggested that pharmacological inhibitors of ATP breakdown in the plasma and of ATP uptake by the lungs may be useful as vasodilators.

^{14}C-adenosine added to whole blood was rapidly taken up by the formed elements and converted to intracellular adenine nucleotides, predominantly ATP. ^{14}C-AMP activity was also identified in the platelet-free plasma of the ^{14}C-adenosine-treated whole blood, and a slight plasma ^{14}C-ATP activity was observed in both control and EDTA-treated bloods. Therefore, the existence of AMP, ATP or other adenine compounds in normal arterial plasma is still questionable.

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