FORMATION AND BREAKDOWN OF ADENOSINE IN THE HEART

Investigations on myocardial purine metabolism

FORMATION AND BREAKDOWN OF ADENOSINE IN THE HEART

Investigations on myocardial purine metabolism

VORMING EN AFBRAAK VAN ADENOSINE IN HET HART

Onderzoekingen aan het myocardiale purine metabolisme

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 15 OKTOBER 1986 OM 16.00 UUR

DOOR

PIETER WILLEM ACHTERBERG

geboren te Zeist

PROMOTIECOMMISSIE

PROMOTOR:	Prof P.G. Hugenholtz
OVERIGE LEDEN:	Prof. Dr. W.C. Hülsmann Prof. Dr. R.S. Reneman Prof. Dr. W. Schaper
CO-PROMOTOR:	Dr. J.W. de Jong

Dit proefschrift kwam mede tot stand dankzij financiële steun van de Nederlandse Hartstichting.

.....Waar of niet waar,
mij raakt het, mij maakt het verzot
met huid en haar op hart en geest
van de weeromstuit, en wel het meest
op wat nog rest aan wijn, aan taal,
dat ik een oud hart nog ophaal
en als een druiventros uitpers
tot in de spieren van het vers.

ARS POETICA (A. Roland Holst, 1960).

AAN MIJN OUDERS

CONTENTS

SUMMARY

Adenosine, a strong coronary vasodilator, is a breakdown product of the myocardial high-energy phosphate ATP. ATP serves as the direct energy source for contraction of the heart. Chapter 1 of this thesis gives a general introduction on contractility dependent ATP-breakdown, the ATP-generating metabolism of the heart and the mechanism which leads to adenosine formation. The applications of purine biochemistry to cardiovascular research are summarized.

In Chapter 2 the main evidence is given for the role of adenosine in the regulation of coronary vasodilation, in mediation of anticatecholaminergic effects as well as for the influence of adenosine on the electromechanical activity of the heart (2.1-2.3). The metabolism of adenosine, adenine nucleotides and other purines in the heart is summarized with emphasis on current clinical applications for diagnostic and therapeutic purposes. The potential use of purines for secondary prevention in clinical cardiology is discussed in relation to the experimental evidence from the literature.
There is considerable evidence of a direct relationship between ischemic release of adenosine and other purines, with the subsequent post-ischemic ATP-content and the function of the heart. This can lead to new ways of therapy, e.g., intracoronary infusion of low doses of ATP or adenosine in the post-ischemic heart (2.4, 2.5).

The experimental section (Chapter 3 and Appendix Papers) gives details on purine metabolism in isolated perfused rat hearts under normoxic and ischemic conditions.
Adenosine, infused into isolated hearts in micromolar concentrations is partly (15-20%) taken up by the heart, but mainly deaminated to inosine and broken down further to hypoxanthine, xanthine and urate. Urate is the major purine (70%) released from the normoxic rat heart. The maximal activity in rat heart homogenates of the enzyme responsible for the formation of urate (xanthine oxidase/dehydrogenase) correlates well with maximal rates of xanthine and urate formation in isolated ischemic hearts. Part of the enzyme is present in the oxidase form, and is capable of

generating free radicals, which can induce irreversible damage to the heart.

The in vitro characteristics of other enzymes of purine metabolism (AMP-deaminase and S-adenosylhomocysteine hydrolase) are reported. Additional perfusion studies with specific inhibitors suggest a significant contribution by these enzymes to myocardial purine formation during normoxia (Appendix Papers II and IV). Several lines of evidence suggest that the purines released from isolated rat hearts are in part degradation products of IMP or GMP instead of AMP. This appears to be especially so during normoxia.

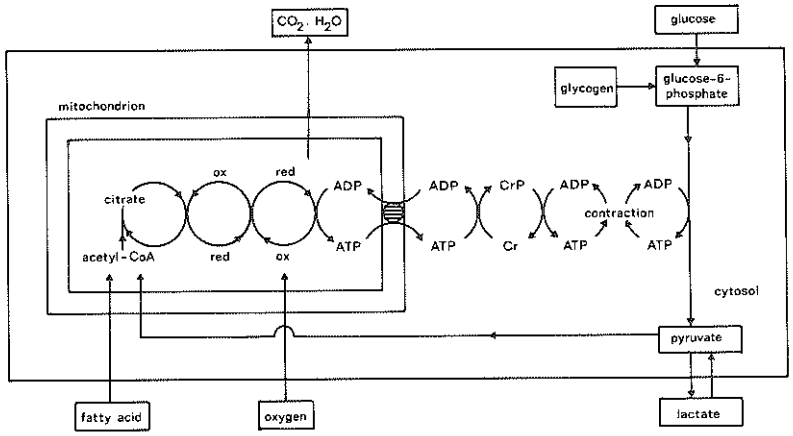
If hypoxanthine or inosine are added to the myocardial perfusate, they are slowly incorporated into the ATP-pool of the heart. The incorporation rate is increased after a previous ischemic period and can be further stimulated by simultaneous infusion of ribose.

Evidence is presented that increased adenosine formation is directly related to increased formation of AMP (e.g., from ATP). The old hypothesis that adenosine is constantly produced at a high rate and nearly simultaneously reincorporated into AMP appears to be unrealistic. The results suggest an even tighter coupling of adenosine and purine release to the myocardial energy state than had already been assumed. A minor disbalance between ATP-breakdown and ATP-production will lead to increased adenosine and purine release and will eventually lead to significant loss of ATP and myocardial function. Some of the experiments point to ways of breaking through this vicious circle by defining conditions for enhanced myocardial ATP-repletion and possible restoration of function during reperfusion.

Chapter 1. PURINE METABOLISM AND THE HEART. GENERAL INTRODUCTION

The energy that is needed for the continuous contraction of the heart is derived from the breakdown of ATP to ADP. Under aerobic conditions the major part of ATP is produced by oxygen-dependent catabolism of nutrients in the mitochondria (see figure 1). Mitochondrial ATP is transported via the adenine nucleotide translocator into the cytosol, where it can be used by the contractile proteins.

FIG. 1 MAJOR PATHWAYS OF ATP-FORMATION FOR MYOCARDIAL CONTRACTION



ATP is produced in the mitochondria by oxidative phosphorylation and transported to the cytosol via the so-called nucleotide translocator. Immediate transfer of the "high-energy" phosphate group of ATP to creatine phosphate (CrP) can occur via the mitochondrion bound enzyme: creatine phosphate kinase (CPK). Cytosolic CPK can produce ATP, which can be used for myocardial contraction. Fatty acids, glucose and lactate are substrates for the heart in the presence of sufficient amounts of oxygen. During hypoxia or ischemia lactate is released from the heart and ATP-production cannot take place sufficiently via anaerobic glycolysis.

The heart supplies the other tissues with the prerequisites of normal cellular function by pumping oxygen-rich and nutrient-containing blood through the body. The heart muscle itself receives oxygen and nutrients via the coronary circulation. A delicate equilibrium exists between myocardial oxygen use (for contraction), oxygen supply and coronary flow. This equilibrium can be regulated by reducing the amount of work performed by the heart

(ATP-breakdown) or by increasing the amount of coronary blood and oxygen that is supplied to the heart. Breakdown of ATP to ADP in the heart during contraction occurs at a very fast rate. About 4-8% of the total ATP (20-25 μmol/g dry wt) is broken down to ADP during a single contraction (59). The ADP formed can be reconverted back to ATP through the action of creatine kinase with breakdown of creatine phosphate to creatine. In this way creatine phosphate provides a temporary buffer against rapid ATP-depletion (Figure 1). Elevated ADP levels can also be reconverted to ATP by the enzyme adenylate kinase with simultaneous formation of AMP. This AMP can be deaminated to IMP (a major pathway in skeletal muscle), but it can also be dephosphorylated to adenosine, which is a potent coronary vasodilator (for details see Chapter 3.1). The phosphorylated, negatively charged purine nucleotides (ATP, ADP, AMP, IMP) will not readily pass through the cell membrane, but adenosine and other non- phosphorylated purines will.

As soon as ATP-breakdown exceeds ATP-production, adenosine and other purines are released from the heart. Their appearance in the coronary circulation correlates with increased coronary flow and thereby increased oxygen supply to the heart. This can enhance ATP-formation and decrease adenosine release and coronary flow. The experimental evidence for the role of adenosine in coronary flow regulation is described in more detail in Chapter 2.2. Adenosine is broken down quickly to inosine and further to hypoxanthine, xanthine and urate, which will also appear in the coronary effluent. The more intricate details of purine metabolism are discussed in Chapter 3.

These general features of myocardial adenine nucleotide and purine metabolism have been studied in more detail for the following reasons:

1) Knowledge of the detailed mechanism of formation and breakdown of adenosine in the heart can provide insight into the regulation of coronary blood flow. In addition, several other physiological effects of adenosine on the heart have been documented. There is evidence of attenuation by adenosine of the deleterious effects of catecholamines in the ischemic heart as well as electrophysiological influences of adenosine

and ATP, both in clinical and in experimental studies (see Chapter 2).

2) The release of adenosine and its breakdown products (i.e., inosine, hypoxanthine, xanthine and urate) can be used to get an indication of the energy state of the heart. Similarly, determination of ATP and creatine phosphate concentrations in heart tissues, for example in peri-operative needle biopsies, can be used for the assessment of the "energetic quality" of heart tissue. Both in experimental and clinical studies, the increased release of purines is a good indicator of myocardial hypoxia or ischemia (Chapter 2.4).
Pharmacologic agents that were found to protect the ischemic heart cause a decrease in ischemic purine release. Calcium antagonists can reduce net ATP-breakdown and purine release by either increasing ATP-formation through their coronary vasodilatory action or by decreasing ATP-breakdown through reduction of the afterload of the heart or through their negative inotropic effect (32, 34, 80). Beta-blockers can cause a decrease in purine release (80, 207), which has been increased by catecholamines. Determination of altered purine release is therefore a valuable tool in pharmacological studies of the heart.

3) Several of the purines mentioned above (adenosine, inosine, hypoxanthine) can be incorporated into the myocardial ATP-pool, which will be severely depleted after prolonged ischemia. The ATP-level after hypoxia and reperfusion of the heart has been shown to correlate well with myocardial function (see Chapter 2.5). Stimulation of the resynthesis of the myocardial ATP-pool by giving purines to the heart could be a factor in promoting the velocity and quality of post-ischemic cardiac recovery. It could also provide additional protection against a lethal second ischemic attack. Moreover, myocardial protection or preservation during open-heart surgery or heart transplantation could also gain from well-defined purine administration, or prevention of purine release.

Chapter 2. PHYSIOLOGICAL IMPORTANCE OF MYOCARDIAL PURINE METABOLISM

2.1. Historical introduction

As early as 1929 Drury and Szent-Györgyi reported the coronary dilatory, and negative chronotropic and dromotropic actions of adenosine on the dog heart (42). A year later adenosine effects on the human heart were reported (84). Effects of adenosine and related compounds on coronary flow of rabbit and dog hearts (213, 214) were described in the early 1930's. The vasodilatory action of purine and pyrimidine nucleotides was studied further (58) and an attempt was made to correlate muscle blood flow and adenine nucleotides in 1932 (160). In later years the coronary dilator properties of purine derivatives (55, 218) and of the "adenine-ATP" series (217) were investigated. The effect of ATP-infusion on the human electrocardiogram was reported in 1949 (212) and in 1955 ATP was found to be effective in the treatment of paroxysmal tachycardia (105, 193).

Around 1960 the changes in myocardial adenine nucleotides and creatine phosphate during hypoxia were described in more detail (15, 130). Then in 1963 two articles appeared (by Berne and Gerlach and coworkers (16, 67)), which mentioned in their title the possible relationship between myocardial adenine nucleotide breakdown and coronary vasodilation. Berne's article (16) schematically introduced the hypothetical role of adenosine in the regulation of coronary flow. Since then numerous studies have been performed to elucidate the qualitative and quantitative aspects of the effects of adenosine on contractility, coronary flow, electrophysiology and biochemistry of the heart (for reviews see: 13, 17, 18, 150, 165, 178, 180, 195). In addition, adenosine has been found to influence various physiological processes in many other tissues. Examples of adenosine-related processes are: blood flow in skeletal muscle, liver, kidney, and brain, the rate of lipolysis in adipose tissue, neurotransmission in the brain, red cell production and platelet aggregation in blood and steroid production by adrenal cells (14, 106, 126, 150, 172, 184, 191).

The following chapter summarizes the physiological effects of

adenosine on the heart as well as the role of adenosine, ATP and other purines in experimental and clinical cardiology. Emphasis is given to the relation between myocardial function and the ATP-content of the heart.

2.2. Adenosine and coronary vasodilation

Numerous experiments have been performed to further study the relationship between adenosine production and coronary vasodilation. Intracoronary infusion of adenosine (8, 25, 115, 169, 176) or ATP, ADP or AMP causes dose-dependent vasodilation. The vasodilation caused by the adenine nucleotides results from their extremely rapid breakdown to adenosine (161, 192). However, this is no proof of coronary flow regulation by endogenous adenosine formation. Several physiologically relevant hemodynamic states in which coronary flow is increased have been experimentally induced and simultaneous changes in adenosine concentration have been determined, e.g., in perfusates of isolated perfused hearts, in coronary sinus whole blood or plasma, in myocardial biopsies, in freeze-clamped hearts, and in pericardial fluid (5, 7, 28, 43, 77, 80, 101, 113, 148).

Hypoxia, reperfusion after ischemia (i.e., reactive hyperemia) and states of increased cardiac work are known to enhance coronary flow rates and adenosine release (7, 8, 40, 57, 80, 127, 133, 151, 194). Hypoxia can be induced by replacing oxygen in blood or perfusate by nitrogen or by infusion of inhibitors of mitochondrial function and oxygen use (cyanide, Amytal) (145, 159).

Increase of myocardial work load can be achieved in vivo by increasing the afterload of the heart via aortic constriction or in isolated hearts by elevating perfusion pressure, and by increasing the heart rate through electrical stimulation (pacing) or by stellate ganglion stimulation (121, 131). Conscious ambulatory animals can be forced to increase total body and heart work in a treadmill or to just increase heart rate in response to a loud noise stress (5, 61). Infusion of catecholamines (epinephrine, norepinephrine), histamine or adrenergic drugs (isoproterenol,

dobutamide) elevate myocardial work and so does infusion of nicotine (50, 215) or calcium at higher than physiological concentrations (35). Under all these conditions of stimulated heart work and consequently stimulated ATP-breakdown, good correlations were observed between coronary flow and adenosine production, either determined in heart tissue or in the coronary fluid (101, 113, 127). In several instances similarly good correlations were observed between myocardial oxygen consumption and coronary blood flow (or coronary vascular resistance) and also between oxygen consumption and adenosine formation (44, 101, 102, 131, 168, 209).

Still all these correlations might point only to a coincidental instead of a causal relationship between adenosine release and coronary vasodilation. No evidence, however, was found for the role of adenosine in the maintenance of an arteriolar vasodilation distal to a coronary stenosis (69); a few authors failed to observe the relation between adenosine concentration and coronary flow (41). The technical problems of adenosine determination could have contributed to these negative results (44, 77, 113).

A second group of experiments which should be mentioned concerns the changing of the adenosine concentration or production in the heart and coronary arteries, either by infusion of adenosine, or by manipulations which alter the adenosine concentration, e.g., by stimulating or preventing the breakdown or by increasing its rate of formation, independent of increased work. A dose-dependent relationship between myocardial adenosine infusion and coronary flow was observed (170, 176). During postischemic reactive hyperemia, adenosine infusion can further increase the coronary flow (if submaximal). Infusion of the enzyme adenosine deaminase, which breaks down adenosine, reduces the extent of the hyperemic flow (169). Modified low molecular weight adenosine deaminase has been used to potentiate its capacity to penetrate the interstitial space (166). The enzyme was found to decrease coronary flow during hypoxia, with simultaneous significant reduction of adenosine in the heart (166). Substances which can be broken down to adenosine in the extracellular space (ATP, ADP, AMP) also cause coronary vasodilation.

Administration of dipyridamole to the hypoxic isolated dog heart results in an increase of tissue adenosine levels without a simultaneous increase in adenosine release (107). This appears to contradict the hypothesis that increased adenosine release is responsible for coronary vasodilation. Later it has been demonstrated, however, that dipyridamole blocks uptake of released adenosine and transport to the vascular space (28). Accumulation of adenosine in the interstitial fluid caused by dipyridamole has been demonstrated by measuring adenosine in pericardial infusates (102). The relationship between coronary blood flow and adenosine release finally appears unchanged by dipyridamole (102). Lidoflazine and mioflazine are chemically related drugs which, like dipyridamole, interact with myocardial adenosine sequestration (52, 91). Lidoflazine was described as an "adenosine-potentiator" and found to enhance coronary reactive hyperemia in conscious pigs (91). Administration of these drugs leads to large increases in myocardial adenosine concentration and increases postischemic function (52).

Not all coronary vasodilators act through an adenosine-related mechanism (e.g., papaverine) (170). For nitroglycerin it has been found that vasodilation was not paralleled by adenosine release; moreover, adenosine is more potent at small than at large coronary vessels, while the reverse is true for nitroglycerin (171).

A special case is coronary vasodilation caused by acetate. Acetate is rapidly taken up by the heart (201) and converted to acetyl-CoA. This "activation" of acetate is an energy-requiring process during which ATP is converted to AMP. Increased AMP levels and coronary flow have been found in isolated hearts given acetate (216). AMP is a direct precursor of adenosine, which will be broken down quickly to inosine and further to (hypo)xanthine and urate. We found a dose-dependent linear increase in total purine formation by acetate (1-20 mM) infused into isolated rat hearts, with a simultaneous increase in coronary flow, without significant changes in cardiac function (Appendix Paper VI). Arterial infusion of acetate in dogs results in increased release of purines from the coronary sinus with increased coronary flow (118). Again, however, the full causal relationship between adenosine and vasodilation is

not complete. Therefore a clear picture of the underlying mechanism(s) of action is needed as well as a more quantitative description of the regulation of formation and breakdown of adenosine. As already mentioned, the determination of adenosine in blood and tissue has several technical pitfalls (44, 80, 113), which makes it extremely difficult to compute actual adenosine concentrations at the site of action from amounts (pmoles, umoles) found in tissue or blood (194).

The first step in the determination of the mechanism of action of adenosine involved the finding of vasodilation resulting from infusion of large molecules (proteins) to which adenosine was chemically bound and which could not penetrate the cell (146, 147, 177). Coronary vasodilation has been elicited by an AMP-protein conjugate (177), by adenosine linked to polysaccharide (146), and to polylysine (147). These conjugates are not taken up by the heart. All these experiments suggest that vasodilation by adenosine is a receptor-mediated process in which the extracellular adenosine concentration determines coronary smooth muscle relaxation. The properties of the vasodilatory adenosine receptor with regard to stereochemical specificity and character of the binding site have been investigated (114, 149).

2.3. Other cardiac effects of adenosine and possible mechanisms of action

Besides the coronary vasodilatory action of adenosine, Drury and Szent-Györgyi (42) also described other effects of adenosine on the heart. Their observations included inhibition by adenosine of electrical conduction and impulse generation in the atrioventricular and sinus node and depression of contractile force, especially in the atrial muscle.
The negative inotropic effect of adenosine is rather weak in the ventricle and in isolated perfused hearts (47, 129), but can be clearly demonstrated as antagonistic to catecholamine-enhanced contractile activity (9, 175). These anti-catecholaminergic effects of adenosine can be important during ischemia when ischemia-induced catecholamine release will enhance ATP-breakdown and speed up the

decrease of cardiac function. A decrease of heart rate by adenosine (75) can slow down ATP-utilization and delay the onset of irreversible damage.
The inhibition of atrial slow action potentials in guinea pig hearts by adenosine was explained by inhibition of Ca^{2+}-influx (49, 173). The involvement of an extracellular adenosine receptor (or "purinergic" receptor (22, 23)) has been mentioned in the previous paragraph.

The link of the adenosine receptor to adenylate cyclase and thereby to increased cyclic AMP formation has been suggested (for short review: see Schütz 1985 (183)), but is not altogether clear. Inhibition of breakdown of cAMP by intracellular adenosine has also been suggested. This would lead to increased potassium conductance and decreased slow inward current of calcium. The interested reader is referred to a number of articles to form his own opinion on the exact mechanism of the electromechanical action of adenosine on atrial, ventricular, vascular and neural tissue of the heart (9, 11-13, 20, 23, 37, 39, 46, 47, 75, 119, 120, 129, 182, 205).

2.4. Adenosine, ATP and other purines in clinical cardiology

The rapidly increasing knowledge of myocardial purine metabolism has already led to diagnostic and therapeutic applications in clinical cardiology and cardiac surgery. This could lead to other clinical applications in the future. Diagnostic applications will be discussed separately from therapeutical and secondary preventive aspects.

Diagnostics

The myocardial (intracellular) content of ATP and creatine phosphate is a good indicator of ischemia or previous ischemia (6, 26, 27, 38, 74, 94, 97, 134). The efficacy of cardioprotective measures during human aorta-coronary bypass surgery has been assessed by the determination of ATP and creatine phosphate in heart biopsies (53, 97, 204). In similar studies the additional protective effect of calcium antagonists in cardioplegic solutions

was reflected by better myocardial ATP preservation (54). The protective effect of lidoflazine, administered to patients subjected to aorta-coronary bypass surgery, was reflected in better postoperative high-energy phosphate contents and better myocardial function (52). In addition, ATP-determinations could be valuable predictors of the quality of donor hearts used for transplantation. It is tempting to redefine myocardial ischemia as a metabolic state where the ratio of ATP-breakdown over ATP-formation is increased. Even during hypothermic cardiac arrest ATP-decline has been reported (38, 97). Minor decreases in myocardial ATP-content are difficult to detect, however, which can explain the repeated failure to detect significant changes in ATP-levels after short periods of ischemia.

An alternative to determinations of ATP in cardiac biopsies or excised papillary muscle is determination of the released catabolites of ATP into the coronary effluent or their accumulation in the heart. Release of adenosine from the human heart was first described by the group of Fox (48, 60, 61). Other authors have also reported purine release from the human heart under ischemic conditions (96, 109-112). In our laboratory we repeatedly (79, 158, 185) demonstrated increased release of hypoxanthine from human hearts during atrial pacing stress tests (APST) and after percutaneous transluminal coronary angioplasty (PTCA). Kaijser and collaborators (43) determined release of adenosine, inosine and hypoxanthine in patients with ischemic heart disease. As already described, adenosine is rapidly broken down in the heart to inosine and hypoxanthine and, in most species, to xanthine and urate. Determination of hypoxanthine is presumably the most sensitive indicator of human myocardial ischemia, because it is a major purine released from the human heart (43). Urate release is difficult to detect because of the high normal blood urate content. An alternative biochemical marker of ischemia is lactate release. It has been found in dogs that increased hypoxanthine release can occur in situations, where lactate is still being extracted (206). Similarly, in patients after an atrial pacing stress test, hypoxanthine release was still observed when lactate was again extracted by the heart after a temporary release phase (79). A correlation between blood levels of purines and severity of

myocardial disease has been observed (21, 48, 62, 70, 76), but other factors than ischemic myocardial purine release could be relevant to this observation (125, 137, 219).

Therapeutics and prevention

It has been suggested (210) that adenosine is a mediator of the paroxysmal bradycardias and tachycardias, which characterize the "sick-sinus syndrome". The adenosine antagonist theophylline (210) was suggested as a therapeutic agent.
On the other hand ATP, adenosine, and the adenosine re-uptake inhibitor dipyridamole are applied with good results for the prevention and therapy of paroxysmal supraventricular tachycardia (13, 37, 71, 105, 154, 193). Apart from the known electrophysiological effects of adenosine and ATP, elevation of the intracellular ATP-pool could be an additional mechanism of action. If ATP is applied intracoronarily or intravenously, it is rapidly broken down to adenosine (161, 192) and in most species the physiological actions of adenosine are similar to those of ATP (12, 13), although different "purinergic" receptors have been suggested for these substances (22, 23).

Beneficial effects of the adenosine catabolite inosine on the ischemic heart have been reported (36, 95, 189). The exact mode of action is unknown, but coronary vasodilation by inosine has been reported (95) as well as increased insulin levels in blood after inosine administration (189). Elevated insulin will enhance glucose utilization and thereby ATP formation. Enhanced net ATP-formation via the incorporation of hypoxanthine derived from inosine can be another important mode of action (Appendix Paper III).

The xanthine oxidase/dehydrogenase inhibitor allopurinol prevents the degradation of hypoxanthine to xanthine and urate. Its described beneficial action on the heart (2, 24, 116) might therefore derive its effect from the increase of hypoxanthine incorporation into myocardial ATP. A mechanism related to the prevention of free radical-induced cellular damage might also be operative, however, in the case of allopurinol (24, 82, 198).

As already mentioned, lidoflazine, a drug which prevents the release and washout of the ATP-catabolite adenosine from the heart, was found to protect the heart during a peri-operative period (52). The mechanism of action can be considered as preventive, when it acts via acceleration of adenosine incorporation into the myocardial ATP-pool. Additional effects of adenosine elevation in the heart by this drug might on the other hand constitute a therapeutic effect (see Chapter 2.3). The beneficial influence of high doses of adenosine, with or without an inhibitor of adenosine breakdown has been reported in several experimental studies (45, 56, 85, 88, 98, 123, 156, 188).

It should be mentioned that adenosine itself (or ATP) cannot be administered intravenously in high doses. Severe hypotension (90) and possibly renal vasoconstriction will result. Moreover, a role for adenosine in the occurrence of immunosuppression is suggested from the existence of the inherited immunodeficiency syndrome "adenosine deaminase deficiency" (187).

The possible importance of enhanced ATP-repletion by giving purines (adenosine, inosine, hypoxanthine) and substances which might enhance the ATP-forming processes (e.g., ribose) is currently under investigation by numerous groups of researchers, but mostly still in animal models. In order to clarify the importance of ATP-repletion in the post-ischemic heart, we will discuss the relationship between myocardial function and myocardial ATP-content in the next chapter.

2.5. Adenine nucleotides, purine metabolism and myocardial function

The direct relationship between myocardial function, oxygen consumption, coronary flow and adenosine production has been described in a previous paragraph. Because adenosine is an ATP-catabolite, a similar, but more intricate relationship between myocardial ATP-metabolism, function, coronary flow and oxygen consumption appears logical.

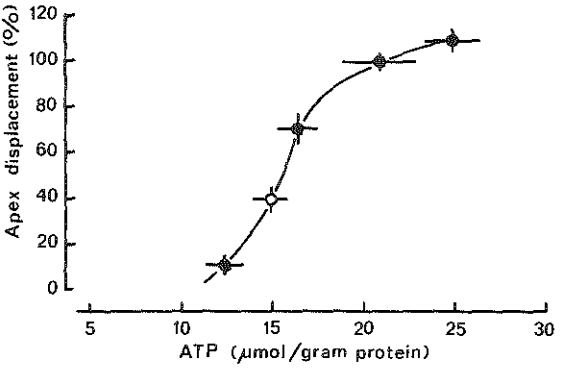
Normoxic myocardial ATP-contents fall in the range between

20-25 μmol/g dry wt. The rates of ATP-breakdown and formation have been estimated between 150 and 600 μmol/g dry wt per minute (81, 103). The extremely high turnover-rate of cardiac ATP, together with compartmentalization of its formation and breakdown make it extremely difficult to adequately describe the relationship between ATP and function in detail. Myocardial purine release, e.g., during prolonged ischemia, hypoxia, increased workload or catecholamine stress, can alter the myocardial adenine nucleotide content considerably (80, 94). In normoxic control hearts or hearts that are reperfused after reversible ischemia or hypoxia, ATP makes up 75 to 80% of the adenine nucleotides (ATP + ADP + AMP). Total adenine nucleotide loss is therefore under these conditions mainly reflected in loss of ATP. The importance of calcium, potassium, sodium, pH and catecholamines as determinants of cardiac contractile function in the non-irreversibly damaged, reperfused heart is beyond question, but there is also considerable evidence that contractile function is directly related to the ATP content of the heart. A short summary of the evidence for a direct relationship between post-reperfusion function of the heart and ATP content will be given.

Coronary artery occlusion of dog hearts in vivo resulted in a more than 50% reduction in the ATP-content of non-infarcted heart muscle and an even greater reduction in infarcted tissue (72-74). Even after ten days the ATP content was still less than 80% of controls in non-infarcted tissue (73). Decreased myocardial function (expressed as stroke work or dP/dt) paralleled ATP-content in these studies. Decreased myocardial ATP contents after short and reversible ischemia in vivo are restored very slowly (27, 87, 94, 122, 157, 199, 200). A similar slow restoration is found for the decreased post-ischemic function (27, 94, 100). In a variety of other studies a good correlation was observed between ATP-content and post-hypoxic or post-ischemic myocardial function, irrespective of the way in which function was determined. Ventricular septal preparations of both newborn and adult rabbit hearts after ischemia (144) or hypoxia (92, 93) followed by reperfusion showed the same correlation, with newborn hearts less susceptible to loss of ATP and loss of function. Isolated, perfused rabbit hearts subjected to different temperatures (hypothermia) during ischemia and protected

with the calcium antagonist nifedipine (138) restored their function in good correlation with the residual ATP-content. Similar results were found with the calcium antagonist verapamil in isolated rat hearts (211). It has been repeatedly shown in our laboratory that several calcium antagonists (i.e., diltiazem, nifedipine, nisoldipine, bepridil) protect the ischemic heart by decreasing ATP-catabolite release (32, 34, 35), which results in better preservation of ATP (80). In earlier studies in our laboratory (196), we have obtained a relation between ATP-content and function in rat hearts subjected to different degrees of hypoxia (Fig. 2). The curve shown is rather similar to other curves obtained after ischemia or hypoxia followed by reperfusion (92, 93, 155).

FIG. 2 RELATION BETWEEN MYOCARDIAL ATP-CONTENT AND CONTRACTILITY



ATP-contents were determined in isolated rat hearts, subjected to varying degrees of hypoxia (●) or ischemia (○). Myocardial function is given as percentage of prehypoxic or pre-ischemic function. Apex displacement is used as a measure of function. Data are redrawn from Stam & De Jong, 1977 (ref 196).

Several authors, however (86, 139, 202), have suggested that there is no linear relationship between myocardial function and ATP-content after severe ischemia and reperfusion. One group (139) presents evidence that ischemic lactate accumulation is an additional important factor which can cause depression of post-ischemic contractile function. Another group (86) negates the ATP-function correlation but simultaneously presents evidence of a

good correlation between ATP-content and recovery of coronary flow in an isolated working heart preparation subjected to ischemia and reperfusion. All find, however, that high ATP levels correlate with good functional recovery and low ATP levels with poor or no functional recovery.

One important implication of the ATP - function relationship is that a heart subjected to a second period of ischemia is bound to completely loose its function, and very fast, if ATP-levels have not been restored in the inter-ischemic period.
A second implication has to do with the observation that ischemic ATP-decline, as for instance during open-heart surgery, is bound to proceed beyond a point of no return. Hypothermia and cardioplegic arrest prevent ischemic ATP-breakdown to a large extent. Still significant ATP-decline can proceed during prolonged hypothermic arrest (83), post-operative function of the heart declines, while sensitivity to a period of accelerated ATP-breakdown will be increased (26, 94, 97).
Considerable experimental effort has therefore been centered on ways to speed up the restoration of the myocardial ATP-content during reperfusion. Incorporation of hypoxanthine (inosine) and adenosine (ATP) are studied most in this respect (4, 36, 45, 56, 83, 85, 88, 89, 95, 98, 123, 124, 156, 188, 189, 208, 220).
A discouraging observation, however, was recently reported by the group of Schaper (83), who observed in intact dog preparations made ischemic by coronary occlusion, that accelerated increase of the post-ischemic myocardial ATP-pool by adenosine infusion did not result in better functional recovery. High adenosine concentrations, as found in these hearts could have depressed myocardial function. Another negative aspect of adenosine administration could be the fact that this will increase the flux through the xanthine oxidase pathways, with a chance of increased free radical formation (see also Chapter 3.2).

A factor which could influence the rate of ATP-restoration is phosphate depletion during ischemia, because the net reaction for adenosine incorporation is: Adenosine + 3 P_i (i.e., inorganic phosphate) to give ATP. The role of phosphate in myocardial processes has not been systematically studied, although perfusion

without phosphate in the medium (197) results in decreased coronary flow, decreased oxygen consumption and lower levels of ATP and creatine phosphate in the heart. Hypophosphatemia in vivo results in myocardial adenine nucleotide breakdown and loss of function (19, 65). Very recently, evidence was presented that inorganic phosphate could indeed be rate-limiting for ATP-repletion by, for instance, adenosine (51). Earlier studies have suggested a role for inorganic phosphate accumulation in the early "pump failure" of the ischemic heart (108).

The adenine nucleotide levels normally found in in vivo or well- perfused hearts are quite similar in human, pig, dog, rat, rabbit and guinea pig. Moreover, these values appear to be about the maximally obtainable levels (89, 156). The incorporation rate of radioactively labeled adenosine into rat cardiac adenine nucleotides was similar in normoxic hearts and in hearts to previously submitted ischemia (which resulted in a 50% decline in adenine nucleotides) and reperfusion (156). Still, when perfusion with adenosine continued for 5 hours, all hearts were found to contain the same amounts of ATP, ADP and AMP, which were moreover similar to the levels in controls before the 5-hour perfusion period (156).

As an alternative for adenosine infusion, ATP has been used. ATP is broken down quickly to adenosine in the heart (161, 192) and can therefore give high local concentrations of adenosine and phosphate. Beneficial effects of ATP-$MgCl_2$ on the heart during reperfusion after global ischemia have indeed been reported (99, 124). In both studies low doses (<0.1 mM) gave better results than high doses (>1 mM). From these studies it was concluded that "post-treatment with low-dose ATP-$MgCl_2$ may be beneficial in cases following myocardial infarction as an adjunct to thrombolysis or angioplasty".

Some of our experiments (Appendix Papers III & VI) have been concerned with the regeneration of the myocardial ATP-pool. We have found that inosine and hypoxanthine (20 μM) are incorporated into myocardial ATP (and GTP) at a relatively slow rate. After ischemia, the velocity of this process is about doubled and ribose further

stimulates the process (Appendix Paper III). The maximal velocity of hypoxanthine incorporation that we found would restore a 20% depleted ATP-pool in 48 hours. Low concentrations of adenosine are incorporated at a faster rate. At 2 μM adenosine 20% of the adenine nucleotide pool might be restored in 4 hours (Appendix Paper V).

Both the fundamental aspects and clinical possibilities of ATP restoration in the heart deserve much further attention, with adenosine- mediated processes as still the most promising.

Chapter 3. FORMATION AND BREAKDOWN OF ADENOSINE IN THE HEART

3.1. Details of myocardial purine metabolism

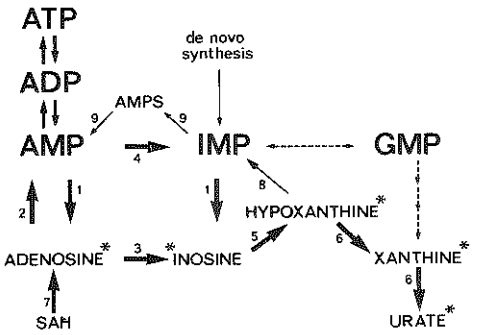
Physiologically relevant adenosine formation in the heart is associated with a disbalance between the very rapid formation and breakdown of ATP (see Chapter 2.2).
It is therefore necessary to study adenosine formation in a preparation (e.g., the isolated, beating heart) which has a considerable rate of ATP-turnover and in which a disbalance of ATP-formation and breakdown can be experimentally induced (e.g., by hypoxia or ischemia). Additional studies on homogenates or purified enzymes from the heart can give insight into the detailed regulation and maximal velocity of certain steps in the metabolic pathways involved.
Possible complications can arise from the fact that the heart consists of several cell types, such as myocytes, fibroblasts, vascular cells, which might have a distinctly different adenosine metabolism. Moreover, it could well be that intracellular compartmentalization of enzymes and substrates (e.g., in mitochondria or cytosol) impedes extrapolation of studies on homogenates to the metabolism of the whole heart.

In this chapter we will describe the major pathways of the myocardial metabolism of purine nucleosides and oxypurines. It will be shown that it is not possible to directly quantify adenosine formation and breakdown, but that several other metabolic pathways influence the quantification of adenosine metabolism, when determined in the perfused heart. Figure 3 summarizes myocardial purine metabolism.

Adenosine formation occurs via dephosphorylation of AMP by the enzyme 5'-nucleotidase or by breakdown of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase. S-adenosylhomocysteine is a product of the cellular methylation cycle (see: Appendix Paper IV). Adenosine can be released from the heart, broken down to inosine by adenosine deaminase, or reincorporated into the adenine nucleotide pool by ATP-dependent phosphorylation to AMP through adenosine

kinase (Fig. 3).
Inosine can be broken down to hypoxanthine by nucleoside phosphorylase. Hypoxanthine can be salvaged by incorporation into IMP (hypoxanthine phosphoribosyltransferase), or broken down to xanthine and urate, both by the enzyme xanthine oxidase/dehydrogenase. Breakdown of IMP or GMP can result in additional release of xanthine and urate. For more elaborate reviews on myocardial and general purine metabolism, see references (62, 80, 94, 122, 132, 152, 178, 180).

FIG. 3 MAJOR PATHWAYS AND ENZYMES OF MYOCARDIAL PURINE METABOLISM



*: Purine nucleosides (adenosine, inosine) and oxypurines (hypoxanthine, xanthine and urate) which can be released into the myocardial perfusate. Enzymes: 1 = 5'-Nucleotidase, 2 = Adenosine Kinase, 3 = Adenosine Deaminase, 4 = AMP-Deaminase, 5 = Nucleoside Phosphorylase, 6 = Xanthine Oxidase/Dehydrogenase, 7 = S-Adenosylhomocysteine Hydrolase, 8 = Hypoxanthine Phosphoribosyl Transferase, SAH = S-Adenosylhomocysteine. IMP can be formed from small precursor molecules by de novo synthesis. Both hypoxanthine salvage and de novo synthesis proceed to AMP via the adenylosuccinate pathway (9). Dotted lines: breakdown of IMP and GMP to xanthine.

The complexity of purine metabolism imposes a number of problems on the study of adenosine formation from determinations of purines in perfusates. Several questions have to be answered, which, moreover, are strongly interrelated. These questions concern the following major topics:

a) How much adenosine catabolites and total purines are released?
b) Are all purines that are released derived from breakdown of adenosine?

c) Is purine release a good measure of total purine and adenosine formation or is the reincorporation of hypoxanthine and adenosine important?

d) The source of adenosine: AMP or S-adenosylhomocysteine?

e) How is increased adenosine formation regulated?

These questions cannot be dealt with simultaneously or in a single experimental approach. In the following paragraphs we will relate our experimental findings to the above described major questions.

3.2. Quantification of all major purines released from the heart The importance of xanthine oxidase/dehydrogenase.

In the past many authors solely determined adenosine, inosine and hypoxanthine in coronary perfusates (7, 8, 174). This is only an adequate measure of purine formation, if no significant amounts of xanthine and urate are formed.

Several lines of investigation have led to a quantification of the importance of urate and xanthine formation in the isolated perfused rat heart under various conditions:

1) Xanthine oxidase/dehydrogenase (XO/XD) activity has been determined in rat heart homogenates. A maximal activity of around 30 nmol/min per g wet weight is found (Appendix Paper I).

2) During normoxia and anoxia hypoxanthine release is significantly increased by infusion of the xanthine oxidase inhibitor allopurinol (Appendix Paper I).

3) Infusion of 8-^{14}C hypoxanthine and 8-^{14}C inosine results in formation of labeled xanthine and urate (Appendix Paper III). From these experiments apparent XO/XD activities of 16-27 nmol/min per g wet wt. can be calculated. Infusion of inosine appears to enhance the apparent XO/XD activity as compared to that found during hypoxanthine infusion and so does the simultaneous infusion of ribose with hypoxanthine (Appendix Paper III).

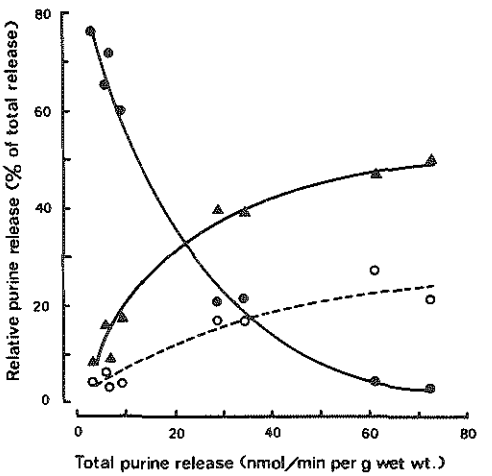
4) Infusion of adenosine also results in formation of xanthine and urate. This has been demonstrated before by others (161). The apparent XO/XD activities that we compute are around 12 nmol/min during 1 μM adenosine infusion and already reach

20 nmol/min per g wet wt at infusion of 5 μM adenosine (Appendix Paper V).

On combining the results we conclude that catabolism of infused adenosine in the rat heart proceeds up to urate at a fast rate, even if low concentrations of adenosine are infused.

The relative importance of purine catabolism up to urate is also apparent from our finding that total purine release from well-oxygenated hearts consists for more than 70% of urate (Figure 4). During ischemia and hypoxia, however, this percentage drops, and adenosine, inosine and hypoxanthine become the main purines that are released (Appendix Paper VI).

FIG. 4 CONTRIBUTION OF ADENOSINE, INOSINE AND URATE TO TOTAL PURINE RELEASE FROM ADULT HEART



Urate (●—●) is the major purine released during normoxia, when total purine release is low. The contribution of inosine (▲—▲) increases during hypoxia and low-flow ischemia, when total release is higher. Adenosine (○—○) increases more slowly than inosine. The contributions of hypoxanthine and xanthine are not shown. These are about equal and never amount to more than 15% each.

Recently, we observed that hearts of newborn rats (10 days old) release about tenfold less urate than adults, presumably because XO/XD activity is significantly lower in these young hearts. Neonatal rat hearts (1-2 days old) have thirtyfold lower

XO/XD activities than adult hearts (B. Schoutsen, personal communication). It appears, moreover, that xanthine oxidoreductase activity is only present in vascular endothelial cells (68). The recent finding (10) that urate is also the major purine (80% of total) released from the normoxic guinea-pig heart corroborates our results in rat hearts and throws a different light on quantitative studies on adenosine formation in this species (8).

Xanthine oxidase/dehydrogenase activity in the heart is mainly present in the dehydrogenase form, which produces NADH from NAD^+ in the course of urate formation. A part of the enzyme (20%) is present in the oxidase form (Appendix Paper I). Xanthine oxidase produces superoxide anion radicals from oxygen. During ischemia part of the dehydrogenase form can be converted into the oxidase form (24). Ischemia and reperfusion provide both substrate (hypoxanthine) and oxygen for the oxidase and increased free radical generation occurs, which causes at least part of the normally observed reperfusion damage (82, 186). The extent of reperfusion damage can be reduced by the xanthine oxidase inhibitor allopurinol. Because the enzyme is strictly located in the endothelium, it appears that reperfusion damage could have the endothelium as a major target (68).

3.3. Are all released purines adenosine catabolites?

If all purines that are released from the heart are adenosine catabolites, quantification of adenosine formation and release would be much simplified.
Purine formation from breakdown of GMP and IMP instead of AMP cannot be excluded as IMP accumulation has been repeatedly described in ischemic or anoxic perfused hearts and in isolated anoxic cardiomyocytes (66, 67, 85, 86, 153). Moreover, in skeletal muscle IMP accumulation is the predominant pathway of ATP catabolism (30, 62) during hypoxia. If IMP is broken down to inosine at a similar rate as its rate of formation, no accumulation will be found in the heart. Our experiments (Appendix Paper II) on purified AMP-deaminase, the enzyme which catalyzes the formation of IMP from AMP, demonstrate a regulation by the adenylate energy charge (3), which is expressed as:

(ATP + ½ADP)/(ATP + ADP + AMP). These results would suggest a very low normoxic rate of IMP-formation in relation to the maximal activity of the enzyme. The maximal activity, however, is several orders of magnitude higher than normoxic purine release. Several other factors (creatine phosphate, inorganic phosphate) (see Appendix Paper III) have been reported to influence AMP-deaminase activity in vitro.
When an inhibitor of adenosine deaminase is infused during ischemia, a large increase in adenosine release is observed (Appendix Paper VI) without alteration of total purine release. Still, adenosine only amounts to 60% of ischemic purine release during infusion of EHNA (Erythro-9-)2-hydroxy-3-nonyl adenine). EHNA is a very effective inhibitor of deamination of infused adenosine. During normoxia EHNA infusion gives only a minor increase in adenosine release. This points to an important contribution of IMP- or GMP-breakdown to normoxic purine release.

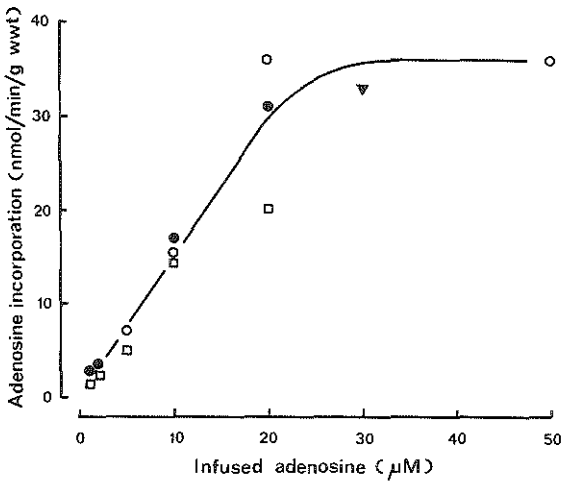
It has been shown that the enzyme, which is most probably responsibe for the breakdown of AMP and IMP (i.e., cytosolic 5'-nucleotidase) dephosphorylates IMP and AMP equally fast (63, 64, 135, 181). The enzyme reaches its half-maximal activity at very low concentrations (5-20 μM) of IMP and AMP (63, 64, 135, 181). Most of the AMP found in acid extracts of heart is presumably bound (to proteins). The actual AMP concentration is calculated (104, 190) at 2-5 micromolar. IMP is present in hearts at 0.1 μmol/g dry wt. or more (86, 203). IMP could therefore be present at several-fold higher free concentrations than AMP and be hydrolyzed at a faster rate during normoxia. Other authors have made similar suggestions for normoxic and ischemic guinea pig hearts (180, 203).
In conclusion: Especially during normoxia, purine release by rat heart is only partly derived from adenosine catabolism.

3.4. Salvage of hypoxanthine and reincorporation of adenosine

The potential importance of restoration of the myocardial ATP-pool and function of the heart via salvage of hypoxanthine or reincorporation of adenosine is described in Chapter 2.5. Infusion of radioactively labeled hypoxanthine and inosine

(Appendix Paper III) results in incorporation of radioactivity in the myocardial adenine nucleotide and guanine nucleotide pool. The incorporation is faster in hearts that have been subjected to a previous period of ischemia as compared to normoxic controls. Simultaneous infusion of ribose stimulates the incorporation further. These incorporation rates are about one order of magnitude lower than the normoxic purine release. Reincorporation of endogenously formed hypoxanthine will therefore not substantially influence the estimation of purine formation and release from the heart.

FIG. 5 ADENOSINE UPTAKE AND INCORPORATION INTO ADENINE NUCLEOTIDES



Incorporation rates of ^{14}C-adenosine into ATP as reported by Namm (□; ref. 136), Aussedat (○; ref. 4) and Newby (▽; ref. 140) and apparent uptake rates as determined by us (●; App. paper V) are linear with the adenosine concentration infused up to 20 - 30 μM. (All experiments in rat hearts).

In the clinical situation, however, a four- to five-fold stimulated recovery rate of myocardial ATP-content could be of enormous significance. In models of cardiac ischemia in the rat (in vivo) the stimulatory effects of inosine and ribose infusion on ATP-regeneration have been repeatedly demonstrated (36, 208, 220). Inosine (Trophicardyl) is used as a cardiotonic drug in some European countries. Good research as to its effects in patients has not been carried out, however.

Adenosine infusion into isolated rat hearts (Appendix Paper V) gives a concentration-dependent uptake of the nucleoside. These uptake rates are similar to the rates of incorporation of radioactive adenosine into adenine nucleotides that were determined by other authors (4, 136, 140, see Figure 5). Several factors determine the incorporation rate of infused adenosine. Apart from possible limitations by transport, infusion of too high concentrations can inhibit adenosine kinase and can have negative hemodynamic and electromechanical effects on the heart. Low concentrations are therefore necessary, but then the actual rate of input at a given coronary flow might be lower than the possible rate of incorporation. The fast deamination (Appendix Paper V) probably gives the most severe restriction of adenosine availability, however.
The use of ATP or AMP as adenosine precursor can possibly lead to high adenosine concentrations near the cellular membrane with a resultant increased uptake (64) as compared to similar concentrations of infused adenosine.

3.5. Two sources of adenosine: S-adenosylhomocysteine and AMP

The constant formation of small amounts of adenosine from S-adenosylhomocysteine in the normoxic rat heart, as suggested from studies which determined the rate of the cellular methylation cycle is confirmed by our experiments with homocysteine infusion (Appendix Paper IV). Although this rate of adenosine formation is only a small portion of total purine release, there are several arguments which enhance its importance. From our perfusion studies with an adenosine deaminase inhibitor (EHNA) it is concluded that a significant part of normoxic purine release is not derived from breakdown of AMP to adenosine (Appendix Paper V). This increases the relative importance of adenosine production from S-adenosylhomocysteine during normoxia. During ischemia, however, adenosine formation from its homocysteine adduct can be neglected. The role of the enzyme in the guinea pig heart appears to be even greater, because the maximal activity of the enzyme is several-fold higher in that species (179). Elevated serum levels of homocysteine, which will block adenosine formation from

S-adenosylhomocysteine occur in certain inborn errors of metabolism (78, 128). An increased incidence of arteriosclerosis has been found in this disease (78, 128). It is interesting to speculate about decreased myocardial adenosine release, possible increased vascular cell stiffness and induction of arteriosclerosis in this disease state (68). Especially since homocysteine-thiolactone infusion is found to reduce myocardial reactive hyperemia, although this last observation has been challenged (167).

Our later experiments with infusion of an adenosine kinase inhibitor suggest (Appendix Paper VI) that adenosine formation from AMP is several-fold higher than adenosine formation in the methylation cycle. A large part of adenosine is immediately reincorporated into AMP. One can argue, however, that the isolated glucose-perfused rat heart will have a worse balance between ATP-formation and ATP-breakdown than the heart in vivo and therefore produce more adenosine from AMP than the latter. The importance of increased AMP-formation for increased adenosine production will be further discussed in paragraph 3.6.

3.6. Adenosine cycling and the regulation of increased adenosine formation

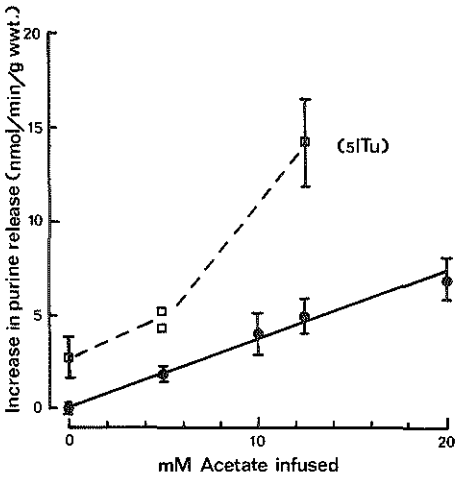
One of the first hypotheses about the regulation of adenosine formation suggested that adenosine was formed at a relatively high rate from AMP and immediately reincorporated again into AMP by adenosine kinase (1, 143). This enzyme has a half-maximal activity at very low concentrations of adenosine (Km: 0.5-1 micromolar) (29, 31). The kinase is inhibited by adenosine concentrations higher than 10 to 20 μM (29, 31, 143). Increased adenosine formation from AMP was assumed to result in increased adenosine release by decreased reincorporation. Because of the rather high maximal activity of adenosine kinase (40-200 nmol/min per g wet wt.) and the fact that adenosine phosphorylation requires ATP, this regulatory mechanism would be energy consuming.

Infusion of a potent inhibitor of adenosine kinase in isolated normoxic rat hearts (140) has been found to result in unchanged

release of adenosine, inosine and hypoxanthine. However, during normoxia, urate accounts for more than 70% of total purines released; effects of the inhibitor can be expected to especially increase urate.

Our experiments with infusion of the adenosine kinase inhibitor (5-iodotubercidin) indeed show an increased total normoxic purine release (Appendix Paper VI). The increase, however, is still very small. The inhibitor does not even double the control release and the effect is a fraction of the maximal adenosine kinase activity. This suggests that the adenosine-cycling hypothesis is not feasible in its originally proposed form. Then how is adenosine formation regulated?

FIG. 6 ACETATE DEPENDENT INCREASE IN NORMOXIC PURINE RELEASE IS ENHANCED BY ADENOSINE KINASE INHIBITION



Acetate infusion causes a dose-dependent increase in total purine release (●—●). Simultaneous infusion of an inhibitor of adenosine reincorporation (5-iodotubercidin, 5ITu) further enhances total purine release (□—□). Control release has been substracted.

Increased AMP-formation is the simplest explanation. This is suggested by the fact that infusion of acetate gives a dose-dependent increase in purine release (Appendix Paper VI, Figure 6). Acetate is rapidly taken up by the heart and transformed

into acetyl-CoA. Acetyl-CoA can be used as a substrate for mitochondrial ATP-formation. This "activation" of acetate requires ATP, and AMP (not ADP!) is formed. Acetate infusion results in increased myocardial AMP-levels (216). Simultaneous infusion of acetate and iodotubercidin, however, gives an increase of purine release which is far more than additive (Appendix Paper VI). This shows that adenosine-cycling can occur and that it can be inhibited by iodotubercidin.

We have already mentioned the fact that normal "free" AMP-concentrations appear to be extremely low (preceding paragraph). In addition, adenylate kinase maintains the following equilibrium: 2 ADP$\rightleftharpoons$ATP + AMP. Because of the fact that the ratio ATP/AMP is normally very high, a small, barely detectable, change in ATP will result in a several-fold increase in AMP and adenosine formation (142). The possible influence of high rates of fatty acid activation on adenosine formation explains the repeated findings of increased coronary flow during perfusion with fatty acids. Adenosine formation, therefore, is very tightly coupled to the equilibrium between formation and breakdown of ATP and to substrate use by the heart.

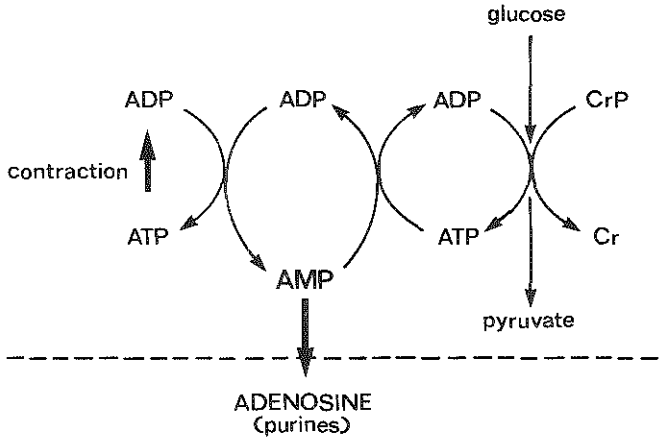
The recent literature on the regulation of adenosine formation in the heart (8, 195) suggests that in physiological terms adenosine formation is regulated by the oxygen supply to demand ratio. In biochemical terms, however, adenosine formation is caused by increased AMP-formation (141), either caused by a decreased ratio of ATP-formation over ATP-breakdown, increased breakdown of cyclic AMP or increased fatty acid activation. Especially the finding of increased purine release and vasodilation during acetate infusion (118, 197) appears to contradict the oxygen supply-demand hypothesis.

3.7. Final conclusions

For the quantification of purine release from isolated rat hearts it is essential to determine the formation rate of xanthine and urate. This is especially so during normoxia and mild ischemia

or hypoxia. This fact has recently been confirmed for guinea pig hearts, but applies to all species with myocardial xanthine oxidase/dehydrogenase activity, including man. The combined evidence that the enzyme responsible for urate formation is localized in the endothelium and that it can generate free radicals during reperfusion makes it necessary to study the role of the endothelium in the genesis of reperfusion damage and possibly atherosclerosis.

FIG. 7 TIGHT COUPLING OF ADENOSINE- AND PURINE-FORMATION TO MYOCARDIAL ATP-TURNOVER



ATP-formation in the contractile compartment is depending mainly on creatine phosphate breakdown to creatine and partly on (anaerobic) breakdown of glucose to pyruvate. ATP-breakdown is mainly depending on contraction-related ATP-ase activity. Adenylate Kinase maintains the equilibrium between ATP, ADP and AMP. Increased AMP-levels will result in increased adenosine and purine formation. Both increased contractile activity (ATP-breakdown) and decreased ATP-formation will, at least temporarily, increase AMP-levels and adenosine formation.

Adenosine formation is tightly coupled to substrate use, ATP-turnover and function of the heart and regulates coronary flow and oxygen supply. The increased formation of adenosine appears to be regulated by the myocardial AMP concentration (Figure 7).

The beneficial effects of stimulated net ATP-synthesis after ischemia by purines deserve much further attention, together with a

investigation of the relation between myocardial ATP, ADP, AMP and function of the heart under various conditions.

A serious search for clinical applications of the accumulated knowledge of myocardial purine metabolism would appear to be unavoidable.

R E F E R E N C E S

REFERENCES TO CHAPTERS 1-3

1. Arch, JRS, Newsholme, EA.
The control of the metabolism and the hormonal role of adenosine.
Essays Biochem (1972) 14, 82-123.
2. Arnold, WL, DeWall, RA, Kezdi, P, Zwart, HHJ.
The effect of allopurinol on the degree of early myocardial ischemia.
Am Heart J (1980) 99, 614-624.
3. Atkinson, DE, Walton GM.
Adenosine triphosphate conservation in metabolic regulation.
J Biol Chem (1967) 242, 3239-3241.
4. Aussedat, J, Verdys, M, Rossi, A.
Synthèse des nucléotides adényliques à partir de l'adénosine exogène dans le coeur de rat perfusé dans des conditions de normoxie et après ischémie.
Arch Int Physiol Biochim (1984) 92, 203-217.
5. Bacchus, AN, Ely, SW, Knabb, RM, Rubio, R, Berne, RM.
Adenosine and coronary blood flow in conscious dogs during normal physiological stimuli.
Am J Physiol (1982) 243, H628-H633.
6. Balderman, SC, Bhayana, JN, Binette, P, Chan, A, Gage, AA.
Perioperative preservation of myocardial ultrastructure and high-energy phosphates in man.
J Thorac Cardiovasc Surg (1981) 82, 860-869.
7. Bardenheuer, H, Schrader, J.
Relationship between myocardial oxygen consumption, coronary flow, and adenosine release in an improved isolated working heart preparation of guinea pigs.
Circ Res (1983) 51, 263-271.
8. Bardenheuer, H, Schrader, J.
Supply-to-demand ratio for oxygen determines formation of adenosine by the heart.
Am J Physiol (1986) 250, H173-H180.
9. Baumann, G, Schrader, J, Gerlach, E.
Inhibitory action of adenosine on histamine- and dopamine-stimulated cardiac contractility and adenylate cyclase in guinea pigs.
Circ Res (1981) 48, 259-266.
10. Becker, BF, Gerlach, E.
Uric acid, the major adenine nucleotide catabolite released from isolated perfused guinea pig hearts, is formed in the coronary endothelium.
J Mol Cell Cardiol (1986) 18, Suppl 1 (Abstr 157).
11. Belardinelli, L, Mattos, EC, Berne, RM.
Evidence for adenosine mediation of atrioventricular block in the ischemic canine myocardium.
J Clin Invest (1981) 68, 195-205.
12. Belhassen, B, Pelleg, A, Shoshani, D, Geva, B, Laniado, S.
Electrophysiologic effects of adenosine-5'-triphosphate on atrioventricular reentrant tachycardia.
Circulation (1983) 68, 827-833.
13. Belhassen, B, Pelleg, A.
Electrophysiologic effects of adenosine triphosphate and adenosine on the mammalian heart: clinical and experimental aspects.
J Am Coll Cardiol (1984) 4, 414-424.
14. Belloni, FL, Phair, RD, Sparks, HV.
The role of adenosine in prolonged vasodilation following flow-restricted exercise of canine skeletal muscle.
Circ Res (1979) 44, 759-766.

15. Benson, ES, Evans, GT, Hallaway, BE, Phibbs, C, Freier, EF.
Myocardial creatine phosphate and nucleotides in anoxic cardiac arrest and recovery.
Am J Physiol (1961) 201, 687-693.
16. Berne, RM.
Cardiac nucleotides in hypoxia: Possible role in regulation of coronary blood flow.
Am J Physiol (1963) 204, 317-322.
17. Berne, RM, Rubio, R.
Role of adenosine, adenosine triphosphate and inorganic phosphate in resistance vessel vasodilatation.
In: Mechanisms of vasodilatation. PM Vanhoutte, I Leusen (Eds.). Karger, Basel (1978), pp 214-221.
18. Berne, RM.
The role of adenosine in the regulation of coronary blood flow.
Circ Res (1980) 47, 807-813.
19. Brautbar, N, Baczynski, R, Carpenter, C, Moser, S, Geiger, P, Finander, P, Massry, S.G.
Impaired energy metabolism in rat myocardium during phosphate depletion.
Am J Physiol (1982) 242, F699-F704.
20. Brückner, R, Fenner, A, Meyer, W, Nobis, TM, Schmitz, W, Scholz, H.
Cardiac effects of adenosine and adenosine analogs in guinea-pig atrial and ventricular preparations: Evidence against a role of cyclic AMP and cyclic GMP.
J Pharmacol Exp Ther (1985) 234, 766-774.
21. Buhl, L, Vilhelmsen, KN, Rokkedal Nielsen, J.
Oxypurine release in cardiac disease.
Acta Med Scand (1981) 209, 83-86.
22. Burnstock, G.
Purinergic receptors in the heart.
Circ Res (1980) 46, I175-I182.
23. Burnstock, G, Meghji, P.
The effect of adenyl compounds on the rat heart.
Br J Pharmacol (1983) 79, 211-218.
24. Chambers, DE, Parks, DA, Patterson, G, Roy, R, McCord, JM, Yoshida, S, Parmley, LF, Downey, JM.
Xanthine oxidase as a source of free radical damage in myocardial ischemia.
J Mol Cell Cardiol (1985) 17, 145-152.
25. Crystal, GJ, Downey, HF, Bashour, FA.
Persistent coronary vasodilation during long-term, supramaximal doses of adenosine.
Am J Physiol (1984) 247, H869-H873.
26. Cunningham, JN, Adams, PX, Knopp, EA, Baumann, FG, Snively, SL, Gross, RI, Nathan, IM, Spencer, FC.
Preservation of ATP, ultrastructure, and ventricular function after aortic cross-clamping and reperfusion.
J Thorac Cardiovasc Surg (1979) 78, 708-720.
27. DeBoer, LWV, Ingwall, JS, Kloner, RA, Braunwald, E.
Prolonged derangements of canine myocardial purine metabolism after a brief coronary artery occlusion not associated with anatomic evidence of necrosis.
Proc Natl Acad Sci USA (1980) 77, 5471-5475.
28. Degenring, FH, Curnish, RR, Rubio, R, Berne, RM.
Effect of dipyridamole on myocardial adenosine metabolism and coronary flow in hypoxia and reactive hyperemia in the isolated perfused guinea pig heart.
J Mol Cell Cardiol (1976) 8, 877-888.

29. De Jong, JW, Kalkman, C.
Myocardial adenosine kinase: activity and localization determined with rapid, radiometric assay.
Biochim Biophys Acta (1973) 320, 388-396.
30. De Jong, JW,
Biochemistry of acutely ischemic myocardium.
In: The Pathophysiology of Myocardial Perfusion. W Schaper (Ed).
Elsevier/North-Holland Biomed Press, Amsterdam (1979), pp 719-750.
31. De Jong, JW, Keijzer, E, Uitendaal, MP, Harmsen, E.
Further purification of adenosine kinase from rat heart using affinity and ion-exchange chromatography.
Anal Biochem (1980) 101, 407-412.
32. De Jong, JW, Harmsen, E, De Tombe, PP, Keijzer, E.
Nifedipine reduces adenine nucleotide breakdown in ischemic rat heart.
Eur J Pharmacol (1982) 81, 89-96.
33. De Jong, JW, Harmsen, E, Serruys, PW.
Biochemische signalen voor myocardischemie.
Hart Bull (1983) 14, 177-182.
34. De Jong, JW, Harmsen, E, De Tombe, PP.
Diltiazem administered before or during myocardial ischemia decreases adenine nucleotide catabolism.
J Mol Cell Cardiol (1984) 16, 363-370.
35. De Jong, JW, Huizer, T, Tijssen, JGP.
Energy conservation by nisoldipine in ischaemic heart.
Br J Pharmacol (1984) 83, 943-949.
36. DeWitt, DF, Jochim, KE, Behrendt, DM.
Nucleotide degradation and functional impairment during cardioplegia: Amelioration by inosine.
Circulation (1983) 67, 171-178.
37. DiMarco, JP, Sellers, TD, Berne, RM, West, GA, Belardinelli, L.
Adenosine: electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia.
Circulation (1983) 68, 1254-1263.
38. Dobbs, WA, Engelman, RM, Rousou, JH, Pels, MA, Alvarez, JM.
Residual metabolism of the hypothermic-arrested pig heart.
J Surg Res (1981) 31, 319-323.
39. Dobson, JG, Jr.
Mechanism of adenosine inhibition of catecholamine-induced responses in heart.
Circ Res (1983) 52, 151-160.
40. Dole, WP, Yamada, N, Bishop, VS, Olsson, RA.
Role of adenosine in coronary blood flow regulation after reductions in perfusion pressure.
Circ Res (1985) 56, 517-524.
41. Downey, HF, Crystal, GJ, Bockman, EL, Bashour, FA.
Nonischemic myocardial hypoxia: coronary dilation without increased tissue adenosine.
Am J Physiol (1982) 243, H512-H516.
42. Drury, AN, Szent-Györgyi, A.
The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart.
J Physiol London (1929/1930) 68, 213-237.
43. Edlund, A, Berglund, B, Van Dorne, D, Kaijser, L, Nowak, J, Patrono, C, Sollevi, A, Wennmalm, A.
Coronary flow regulation in patients with ischemic heart disease: Release of purines and prostacyclin and the effect of inhibitors of prostaglandin formation.
Circulation (1985) 71, 1113-1120.

44. Edwards, NL, Recker, D, Airozo, D, Fox, IH.
Enhanced purine salvage during allopurinol therapy: an important pharmacological property in humans.
J Lab Clin Med (1981) 98, 673-683.
45. Ely, SW, Knabb, RM, Bacchus, AN, Rubio, R, Berne, RM.
Measurements of coronary plasma and pericardial infusate adenosine concentrations during exercise in conscious dog: Relationship to myocardial oxygen consumption and coronary blood flow.
J Mol Cell Cardiol (1983) 15, 673-683.
46. Ely, SW, Mentzer, RM, Lasley, RD, Lee, BK, Berne, RM.
Functional and metabolic evidence of enhanced myocardial tolerance to ischemia and reperfusion with adenosine.
J Thorac Cardiovasc Surg (1985) 90, 549-556.
47. Endoh, M, Yamashita, S.
Adenosine antagonizes the positive inotropic action mediated via beta-, but not alpha-adrenoceptors in the rabbit papillary muscle.
Eur J Pharmacol (1980) 65, 445-448.
48. Evans, DB, Schenden, JA, Bristol, JA.
Adenosine receptors mediating cardiac depression.
Life Sci (1982) 31, 2425-2432.
49. Feit, F, Fox, AC, Nachamie, M, Gross, RI, Snively, SL, Cunningham, JN.
Nucleoside release reflects changing or prolonged myocardial ischemia.
Clin Res (1980) 28, 168A.
50. Fenton, RA, Bruttig, SP, Rubio, R, Berne, RM.
Effect of adenosine on calcium uptake by intact and cultured vascular smooth muscle.
Am J Physiol (1982) 242, H797-H804.
51. Fenton, RA, Dobson, JG.
Nicotine increases heart adenosine release, oxygen consumption, and contractility.
Am J Physiol (1985) 249, H463-H469.
52. Fischer, M, Buchwald, A, Winkler, B, Schaper, W.
Is inorganic phosphate (P_i) a limiting factor for rapid postischemic ATP repletion?
J Mol Cell Cardiol (1986) 18, Suppl 1, (Abstr. 365).
53. Flameng, W, Borgers, M, Van der Vusse, GJ, Demeyere, R, Vandermeersch, E, Thoné, F, Suy, R.
Cardioprotective effects of lidoflazine in extensive aorta-coronary bypass grafting.
J Thorac Cardiovasc Surg (1983) 85, 758-768.
54. Flameng, W, Van der Vusse, GJ, De Meyere, R, Borgers, M, Sergeant, P, Vander Meersch, E, Geboers, J, Suy, R.
Intermittent aortic cross-clamping versus St. Thomas' Hospital cardioplegia in extensive aorta-coronary bypass grafting.
J Thorac Cardiovasc Surg (1984) 88, 164-173.
55. Flameng, W, De Meyere, R, Daenen, W, Sergeant, P, Ngalikpima, V, Geboers, J, Suy, R, Stalpaert, G.
Nifedipine as an adjunct to St. Thomas' Hospital cardioplegia. A double-blind, placebo controlled, randomized clinical trial.
J Thorac Cardiovasc Surg (1986) 91, 723-731.
56. Fleisch, A, Weger, P.
Die gefässerweiternde Wirkung der phosphorylierten Stoffwechselprodukte.
Arch Ges Physiol (1937) 239, 362-369.
57. Foker, JE, Einzig, S, Wang, T, Anderson, RW.
Adenosine metabolism and myocardial preservation.
J Thorac Cardiovasc Surg (1980) 80, 506-516.

58. Foley, DH, Herlihy, JT, Thompson, CI, Rubio, R, Berne, RM.
Increased adenosine formation by rat myocardium with acute aortic constriction.
J Mol Cell Cardiol (1978) 10, 293-300.
59. Folkow, B.
The vasodilator action of adenosine triphosphate.
Acta Physiol Scand (1949) 17, 311-316.
60. Fossel, ET, Morgan, HE, Ingwall, JS.
Measurement of changes in high-energy phosphates in the cardiac cycle by using gated ^{31}P nuclear magnetic resonance.
Proc Natl Acad Sci USA (1980) 77, 3654-3658.
61. Fox, AC, Reed, GE, Glassman, E, Kaltman, AJ, Silk, BB.
Release of adenosine from human hearts during angina induced by rapid atrial pacing.
J Clin Invest (1974) 53, 1447-1457.
62. Fox, AC, Reed, GE, Meilman, H, Silk, BB.
Release of nucleosides from canine and human hearts as an index of prior ischemia.
Am J Cardiol (1979) 43, 52-58.
63. Fox, IH.
Adenosine triphosphate degradation in specific disease.
J Lab Clin Med (1985) 106, 101-110.
64. Frick, GP, Lowenstein, JM.
Studies of 5'-nucleotidase in the perfused rat heart.
J Biol Chem (1976) 251, 6372-6378.
65. Frick, GP, Lowenstein, JM.
Vectorial production of adenosine by 5'-nucleotidase in the perfused rat heart.
J Biol Chem (1978) 253, 1240-1244.
66. Fuller, TJ, Nichols, WW, Brenner, BJ, Peterson, JC.
Reversible depression in myocardial performance in dogs with experimental phosphorus deficiency.
J Clin Invest (1978) 62, 1194-1200.
67. Geisbuhler, T, Altschuld, RA, Trewyn, RW, Ansel, AZ, Lamka, K, Brierley, GP.
Adenine nucleotide metabolism and compartmentalization in isolated adult rat heart cells.
Circ Res (1984) 54, 536-546.
68. Gerlach, E, Deuticke, B, Dreisbach, RH.
Der Nucleotid-Abbau im Herzmuskel bei Sauerstoffmangel und seine mögliche Bedeutung für die Coronardurchblutung.
Naturwissenschaften (1963) 50, 228-229.
69. Gerlach, E, Nees, S, Becker, BF.
The vascular endothelium: a survey of some newly evolving biochemical and physiological features.
Basic Res Cardiol (1985) 80, 459-474.
70. Gewirtz, H, Brautigan, DL, Olsson, RA, Brown, P, Most, AS.
Role of adenosine in the maintenance of coronary vasodilation distal to a severe coronary artery stenosis: observations in conscious domestic swine.
Circ Res (1983) 53, 42-51.
71. Gneushev, ET, Naumova, VV, Bogolovsky, VA.
Content of hypoxanthin in the peripheral venous blood in infarction and ischemia of the myocardium.
Therapevt Arkh USSR (1978) 50, 20-24.
72. Greco, R, Musto, B, Arienzo, V, Alborino, A, Garofalo, S, Marsico, F.
Treatment of paroxysmal supraventricular tachycardia in infancy with digitalis, adenosine-5'-triphosphate, and verapamil: a comparative study.
Circulation (1982) 66, 504-508.

73. Gudbjarnason, S, Braasch, W, Cowan, C, Bing, RJ.
Metabolism of infarcted heart muscle during tissue repair.
Am J Cardiol (1968) 22, 360-369.
74. Gudbjarnason, S, Puri, PS, Mathes, P.
Biochemical changes in non-infarcted heart muscle following myocardial infarction.
J Mol Cell Cardiol (1971) 2, 253-276.
75. Gudbjarnason, S.
Acute alterations in energetics of ischemic heart muscle.
Cardiology (1971/72) 56, 232-244.
76. Haleen, SJ, Evans, DB.
Selective effects of adenosine receptor agonists upon coronary resistance and heart rate in isolated working rabbit hearts.
Life Sci (1985) 36, 127-137.
77. Halpern MJ, Pereira Miguel, MS.
Uric acid and coronary heart disease.
J Am Geriatr Soc (1974) 22, 86-87.
78. Hanley, F, Messina, LM, Baer, RW, Uhlig, PN, Hoffman, JIE.
Direct measurement of left ventricular interstitial adenosine.
Am J Physiol (1983) 245, H327-H335.
79. Harker, LA, Ross, R, Slichter, SJ, Scott, CR.
Homocystine-induced arteriosclerosis: the role of endothelial cell injury and platelet response to its genesis.
J Clin Invest (1976) 58, 731-741.
80. Harmsen, E, De Jong, JW, Serruys, PW.
Hypoxanthine production by ischemic heart demonstrated by high pressure liquid chromatography of blood purine nucleosides and oxypurines.
Clin Chim Acta (1981) 115, 73-84.
81. Harmsen, E.
Myocardial purine metabolism. Aspects of myocardial ATP metabolism and pharmacological interventions.
Thesis, Erasmus University, Rotterdam, 1984.
82. Hearse, DJ.
Oxygen deprivation and early myocardial contractile failure: a reassessment of the possible role of adenosine triphosphate.
Am J Cardiol (1979) 44, 1115-1121.
83. Hess, ML, Manson, NH, Okabe, E.
Involvement of free radicals in the pathophysiology of ischemic heart disease.
Can J Physiol Pharmacol (1982) 60, 1382-1389.
84. Hoffmeister, HM, Mauser, M, Schaper, W.
Effect of adenosine and AICAR on ATP content and regional contractile function in reperfused canine myocardium.
Basic Res Cardiol (1985) 80, 445-458.
85. Honey, RM, Ritchie, WT, Thomson, WAR.
The action of adenosine upon the human heart.
Quart J Med (1930) 23, 485-489.
86. Humphrey, SM, Seelye, RN.
Improved functional recovery of ischemic myocardium by suppression of adenosine catabolism.
J Thorac Cardiovasc Surg (1982) 84, 16-22.
87. Humphrey, SM, Holliss, DG, Seelye, RN.
Myocardial adenine pool depletion and recovery of mechanical function following ischemia.
Am J Physiol (1985) 248, H644-H651.

88. Isselhard, W, Lauterjung, KL, Witte, J, Ban, T, Hübner, G, Giersberg, O, Heugel, E, Hirt, HJ.
Metabolic and structural recovery of left ventricular canine myocardium from regional complete ischemia.
Eur Surg Res (1975) 7, 136-155.
89. Isselhard, W, Eitenmüller, J, Mäurer, W, Welter, H, Reinecke, H.
Myocardial adenine nucleotides after infusion of adenosine.
In: Rec. Adv. Studies on Card. Struct. Metab. Vol. 11, T Kobayashi, T Sano, NS Dhalla (Eds.). Univ Park Press, Baltimore, 1978, pp. 319-323.
90. Isselhard, W, Eitenmüller, J, Mäurer, W, De Vreese, A, Reineke, H, Czerniak, A, Sturz, J, Herb, H-G.
Increase in myocardial adenine nucleotides induced by adenosine. Dosage, mode of application and duration, species differences.
J Mol Cell Cardiol (1980) 12, 619-634.
91. Isselhard, W, Hamaji, M, Mäurer, W, Erkens, H, Welter, H.
Adenosine-induced increase in myocardial adenine nucleotides without adenosine-induced systemic hypotension.
Basic Res Cardiol (1985) 80, 47-57.
92. Jageneau, AHM, Schaper, WKA, Van Gerven, W.
Enhancement of coronary reactive hyperemia in unanaesthetized pigs by an adenosine-potentiator (lidoflazine).
Naunyn-Schmied Arch Pharmakol (1969) 265, 16-23.
93. Jarmakani, JM, Nagatomo, T, Nakazawa, M, Langer, GA.
Effect of hypoxia on myocardial high-energy phosphates in the neonatal mammalian heart.
Am J Physiol (1978) 235, H474-H481.
94. Jarmakani, JM, Nakazawa, M, Nagatomo, T, Langer, GA.
Effect of hypoxia on mechanical function in the neonatal mammalian heart.
Am J Physiol (1978) 235, H469-H474.
95. Jennings, RB, Steenbergen, C.
Nucleotide metabolism and cellular damage in myocardial ischemia.
Ann Rev Physiol (1985) 47, 727-749.
96. Jones, CE.
Vasodilatory action of inosine in the in vivo heart: A dual mechanism.
J Cardiovasc Pharmacol (1982) 4, 1079-1080.
97. Jorgensen, CR, Gobel, FL, Nordstrom, LA, Nelson, RR, From, AHL, Simon, AB.
Coronary sinus hypoxanthine concentration as an indicator of myocardial ischemia (Abstr.).
Circulation (1976) 54 (Suppl. II), II-108.
98. Kaijser, L, Jansson, E, Schmidt, W, Bomfim, V.
Myocardial energy depletion during profound hypothermic cardioplegia for cardiac operations.
J Thorac Cardiovasc Surg (1985) 90, 896-900.
99. Kao, RL, Magovern, GJ.
Prevention of reperfusional damage from ischemic myocardium.
J Thorac Cardiovasc Surg (1986) 91, 106-114.
100. Klay, JW, Chaudry, IH, Geha, AS, Baue, AE.
Improved myocardial performance with infusion of adenosine triphosphate-$MgCl_2$.
Surg Forum (1980) 31, 260-262.
101. Kloner, RA, DeBoer, LWV, Darsee, JR, Ingwall, JS, Hale, S, Tumas, J, Braunwald, E.
Prolonged abnormalities of myocardium salvaged by reperfusion.
Am J Physiol (1981) 241, H591-H599.
102. Knabb, RM, Ely, SW, Bacchus, AN, Rubio, R, Berne, RM.
Consistent parallel relationships among myocardial oxygen consumption, coronary blood flow, and pericardial infusate adenosine concentration with various interventions and beta-blockade in the dog.
Circ Res (1983) 53, 33-41.

103. Knabb, RM, Gidday, JM, Ely, SW, Rubio, R, Berne, RM.
Effects of dipyridamole on myocardial adenosine and active hyperemia.
Am J Physiol (1984) 247, H804-H810.
104. Kobayashi, K, Neely, JR.
Control of maximum rates of glycolysis in rat cardiac muscle.
Circ Res (1979) 44, 166-175.
105. Kohn, MC, Achs, MJ, Garfinkel, D.
Distribution of adenine nucleotides in the perfused rat heart.
Am J Physiol (1977) 232, R158-R163.
106. Komor, K, Garas, Z.
Adenosine triphosphate in paroxysmal tachycardia.
Lancet (1955) 269, 93.
107. Kowal, J, Fiedler, RP.
Adrenal cells in tissue culture. II. Steroidogenic responses to nucleosides and nucleotides.
Endocrinology (1969) 84, 1113-1117.
108. Kübler, W, Spieckermann, PG, Bretschneider, HJ.
Influence of dipyridamol (Persantin) on myocardial adenosine metabolism.
J Mol Cell Cardiol (1970) 1, 23-28.
109. Kübler, W, Katz, AM.
Mechanisms of early "pump" failure of the ischemic heart: possible role of adenosine triphosphate depletion and inorganic phosphate accumulation.
Am J Cardiol (1977) 40, 467-471.
110. Kugler, G.
The effect of nitroglycerin on myocardial release of inosine, hypoxanthine and lactate during pacing-induced angina.
Basic Res Cardiol (1978) 73, 523-533.
111. Kugler, G.
Effect of pindolol on myocardial release of inosine, hypoxanthine and lactate during pacing-induced angina.
J Pharmacol Exp Ther (1979) 209, 185-189.
112. Kugler, G.
Myocardial release of inosine, hypoxanthine and lactate during pacing-induced angina in humans with coronary artery disease.
Eur J Cardiol (1979) 9, 227-240.
113. Kugler, G.
Myocardial release of lactate, inosine, and hypoxanthine during atrial pacing and exercise-induced angina.
Circulation (1979) 59, 43-49.
114. Kusachi, S, Olsson, RA.
Pericardial superfusion to measure cardiac interstitial adenosine concentration.
Am J Physiol (1983) 244, H458-H461.
115. Kusachi, S, Thompson, RD, Olsson, RA.
Ligand selectivity of dog coronary adenosine receptor resembles that of adenylate cyclase stimulatory (R_a) receptors.
J Pharmacol Exp Ther (1983) 227, 316-321.
116. L'Abbate, A, Camici, P, Trivella, MG, Pelosi, G, Davies, GJ, Ballestra, AM, Taddei, I.
Time-dependent response of coronary flow to prolonged adenosine infusion: doubling of peak reactive hyperaemic flow.
Cardiovasc Res (1981) 15, 282-286.
117. Leung, E, Johnston, CI, Woodcock, EA.
Demonstration of specific receptors for adenosine in guinea-pig myocardium.
Clin Exp Pharmacol Physiol (1983) 10, 325-329.
118. Liang, CG, Lowenstein, JM.
Metabolic control of the circulation. Effects of acetate and pyruvate.
J Clin Invest (1978) 62, 1029-1038.

119. Linden, J, Hollen, CE, Patel, A.
The mechanism by which adenosine and cholinergic agents reduce contractility in rat myocardium. Correlation with cyclic adenosine monophosphate and receptor densities.
Circ Res (1985) 56, 728-735.
120. Lohse, MJ, Ukena, D, Schwabe, U.
Adenosine receptors on heart muscle.
Lancet (1984) II, 355.
121. Manfredi, JP, Sparks, HV, Jr.
Adenosine's role in coronary vasodilation induced by atrial pacing and norepinephrine.
Am J Physiol (1982) 243, H536-H545.
122. Manfredi, JP, Holmes, EW.
Purine salvage pathways in myocardium.
Ann Rev Physiol (1985) 47, 691-705.
123. Mauser, M, Hoffmeister, HM, Nienaber, C, Schaper, W.
Influence of ribose, adenosine, and "AICAR" on the rate of myocardial adenosine triphosphate synthesis during reperfusion after coronary artery occlusion in the dog.
Circ Res (1985) 56, 220-230.
124. McDonagh, PF, Laks, H, Chaudry, IH, Baue, AE.
Improved myocardial recovery from ischemia. Treatment with low-dose adenosine triphosphate-magnesium chloride.
Arch Surg (1984) 119, 1379-1384.
125. McEwin, R, McEwin, K, Loudon, B.
Raised serum uric acid levels with myocardial infarction.
Med J Aust (1974) 1, 530-532.
126. McIlwain, H.
Translocation of neural modulators: a second category of nerve signal.
Neurochem Res (1976) 1, 351-368.
127. McKenzie, JE, McCoy, FP, Bockman, EL.
Myocardial adenosine and coronary resistance during increased cardiac performance.
Am J Physiol (1980) 239, H509-H515.
128. McKully, KS.
Homocystein theory of arteriosclerosis: development and current status.
Atherosclerosis Rev (1983) 11, 157-246.
129. Meyer, W, Nose, M, Schmitz, W, Scholz, H.
Adenosine and adenosine analogs inhibit phosphodiesterase activity in the heart.
Naunyn-Schmied Arch Pharmacol (1984) 328, 207-209.
130. Michal, G, Naegle, S, Danforth, WH, Ballard, FB, Bing, RJ.
Metabolic changes in heart muscle during anoxia.
Am J Physiol (1959) 197, 1147-1151.
131. Miller, WL, Belardinelli, L, Bacchus, A, Foley, DH, Rubio, R, Berne, RM.
Canine myocardial adenosine and lactate production, oxygen consumption, and coronary blood flow during stellate ganglia stimulation.
Circ Res (1979) 45, 708-718.
132. Murray, AW.
The biological significance of purine salvage.
Ann Rev Biochem (1971) 40, 811-826.
133. Mustafa, SJ.
Cellular and molecular mechanism(s) of coronary flow regulation by adenosine.
Mol Cell Biochem (1980) 31, 67-87.

134. Myers, CL, Weiss, SJ, Kirsh, MM, Shepard, BM, Shlafer, M.
Effects of supplementing hypothermic crystalloid cardioplegic solution with catalase, superoxide dismutase, allopurinol, or deferoxamine on functional recovery of globally ischemic and reperfused isolated hearts.
J Thorac Cardiovasc Res (1986) 91, 281-289.
135. Naito, Y, Lowenstein, JM.
5'-Nucleotidase from rat heart.
Biochemistry (1981) 20, 5188-5194.
136. Namm, DH.
Myocardial nucleotide synthesis from purine bases and nucleosides.
Circ Res (1973) 33, 686-695.
137. Naumova, VV, Gneushev, ET, Bogoslovsky, VA.
Hypoxanthine content in the peripheral venous blood of patients with ischemic heart disease with attacks of stenocardia and in patients with osteochondrosis of the cervicothoracic region of the vertebral column.
Therapevt Arkh USSR (1979) 51, 22-27.
138. Nayler, WG.
Protection of the myocardium against postischemic reperfusion damage. The combined effect of hypothermia and nifedipine.
J Thorac Cardiovasc Surg (1982) 84, 897-905.
139. Neely, JR, Grotyohann, LW.
Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts.
Circ Res (1984) 55, 816-824.
140. Newby, AC, Holmquist, CA, Illingworth, J, Pearson, JD.
The control of adenosine concentration in polymorphonuclear leucocytes, cultured heart cells and isolated perfused heart from the rat.
Biochem J (1983) 214, 317-323.
141. Newby, AC.
The role of adenosine kinase in regulating adenosine concentration.
Biochem J (1984) 226, 343-344.
142. Newsholme, EA, Start, C.
Regulation in metabolism.
John Wiley & Sons, London (1973), pp 111-113.
143. Newsholme, EA, Fisher, MN.
Adenosine kinase and the control of adenosine concentration in the heart.
Biochem J (1984) 226, 344.
144. Nishioka, K, Jarmakani, JM.
Effect of ischemia on mechanical function and high-energy phosphates in rabbit myocardium.
Am J Physiol (1982) 242, H1077-H1083.
145. Nuutinen, EM, Nelson, D, Wilson, DF, Erecinska, M.
Regulation of coronary blood flow: effects of 2,4-dinitrophenol and theophylline.
Am J Physiol (1983) 244, H396-H405.
146. Olsson, RA, Davis, CJ, Khouri, EM, Patterson, RE.
Evidence for an adenosine receptor on the surface of dog coronary myocytes.
Circ Res (1976) 39, 93-98.
147. Olsson, RA, Davis, CC, Khouri, EM.
Coronary vasoactivity of adenosine covalently linked to polylysine.
Life Sci (1977) 21, 1343-1349.
148. Olsson, RA, Snow, JA, Gentry, MK.
Adenosine metabolism in canine myocardial reactive hyperemia.
Circ Res (1978) 42, 358-362.
149. Olsson, RA, Khouri, EM, Bedynek, JL, McLean, J.
Coronary vasoactivity of adenosine in the conscious dog.
Circ Res (1979) 45, 468-478.

150. Olsson, RA.
Local factors regulating cardiac and skeletal muscle blood flow.
Ann Rev Physiol (1981) 43, 385-395.
151. Onwochei, MO, Mustafa, SJ.
Relationship between coronary flow and adenosine release in reactive hyperemia.
Life Sci (1985) 36, 105-112.
152. Opie, LH.
High energy phosphate compounds.
In: Cardiac metabolism. AJ Drake-Holland, MIM Noble (Eds.), John Wiley & Sons, New York (1983), pp. 279-307.
153. Parker, JC, Smith, EE, Jones, CE.
The role of nucleoside and nucleobase metabolism in myocardial adenine nucleotide regeneration after cardiac arrest.
Circ Shock (1976) 3, 11-20.
154. Pelleg, A, Belhassen, B, Ilia, R, Laniado, S.
Comparative electrophysiologic effects of adenosine triphosphate and adenosine in the canine heart: influence of atropine, propranolol, vagotomy, dipyridamole and aminophylline.
Am J Cardiol (1985) 55, 571-576.
155. Reibel, DK, Rovetto, MJ.
Myocardial ATP synthesis and mechanical function following oxygen deficiency.
Am J Physiol (1978) 234, H620-H624.
156. Reibel, DK, Rovetto, MJ.
Myocardial adenosine salvage rates and restoration of ATP content following ischemia.
Am J Physiol (1979) 237, H247-H252.
157. Reimer, KA, Hill, ML, Jennings, RB.
Prolonged depletion of ATP and of the adenine nucleotide pool due to delayed resynthesis of adenine nucleotides following reversible myocardial ischemic injury in dogs.
J Mol Cell Cardiol (1981) 13, 229-239.
158. Remme, WJ, De Jong, JW, Verdouw, PD.
Effects of pacing-induced myocardial ischemia on hypoxanthine efflux from the human heart.
Am J Cardiol (1977) 40, 55-62.
159. Richman, HG, Wyborny, L.
Adenine nucleotide degradation in the rabbit heart.
Am J Physiol (1964) 207, 1139-1145.
160. Rigler, R.
Über die Ursache der vermehrten Durchblutung des Muskels während der Arbeit.
Arch Exp Pathol Pharmakol (1932) 167, 54-56.
161. Ronca-Testoni, S, Borghini, F.
Degradation of perfused adenine compounds up to uric acid in isolated rat heart.
J Mol Cell Cardiol (1982) 14, 177-180.
162. Rubio, R, Berne, RM.
Release of adenosine by the normal myocardium in dogs and its relationship to the regulation of coronary resistance.
Circ Res (1969) 25, 407-415.
163. Rubio, R, Berne, RM, Katori, M.
Release of adenosine in reactive hyperemia of the dog heart.
Am J Physiol (1969) 216, 56-62.
164. Rubio, R, Wiedmeier, VT, Berne, RM.
Relationship between coronary flow and adenosine production and release.
J Mol Cell Cardiol (1974) 6, 561-566.

165. Rubio, R, Berne, RM.
Regulation of coronary blood flow.
Progr Cardiovasc Dis (1975) 18, 105-122.
166. Rubio, R, Knabb, RM, Ely, SW, Berne, RM.
A 50% decrease in the coronary flow (CF) responses to hypoxia caused by "micro" adenosine deaminases (MADA).
Fed Proc (1982) 41, 1599 (Abstr.).
167. Sadick, N, Campano German, D, McHale, PhA, Greenfield, JC, Kredich, NM.
Functional and reactive hyperemia are unaltered by homocysteine in conscious dogs.
Basic Res Cardiol (1985) 80, 280-290.
168. Saito, D, Nixon, DG, Vomacka, RB, Olsson, RA.
Relationship of cardiac oxygen usage, adenosine content, and coronary resistance in dogs.
Circ Res (1980) 47, 875-882.
169. Saito, D, Steinhart, CR, Nixon, DG, Olsson, RA.
Intracoronary adenosine deaminase reduces canine myocardial reactive hyperemia.
Circ Res (1981) 49, 1262-1267.
170. Saito, D, Hyodo, T, Takeda, K, Abe, Y, Tani, H, Yamada, N, Ueeda, M, Nakatsu, T.
Intracoronary adenosine enhances myocardial reactive hyperemia after brief coronary occlusion.
Am J Physiol (1985) 248, H812-H817.
171. Schnaar, RL, Sparks, HV.
Response of large and small coronary arteries to nitroglycerin, $NaNO_2$, and adenosine.
Am J Physiol (1972) 223, 223-228.
172. Schooley, JC, Mahlmann, LJ.
Adenosine, AMP, cyclic AMP, theophylline and the action and production of erythropoietin (39005).
Proc Soc Exp Biol Med (1975) 150, 215-219.
173. Schrader, J, Rubio, R, Berne, RM.
Inhibition of slow action potentials of guinea pig atrial muscle by adenosine: a possible effect on Ca^{2+} influx.
J Mol Cell Cardiol (1975) 7, 427-433.
174. Schrader, J, Gerlach, E.
Compartmentation of cardiac adenine nucleotides and formation of adenosine.
Pflügers Arch (1976) 367, 129-135.
175. Schrader, J, Baumann, G, Gerlach, E.
Adenosine as inhibitor of myocardial effects of catecholamines.
Pflügers Arch (1977) 372, 29-35.
176. Schrader, J, Haddy, FJ, Gerlach, E.
Release of adenosine, inosine and hypoxanthine from the isolated guinea pig heart during hypoxia, flow-autoregulation and reactive hyperemia.
Pflügers Arch (1977) 369, 1-6.
177. Schrader, J, Nees, S, Gerlach, E.
Evidence for a cell surface adenosine receptor on coronary myocytes and atrial muscle cells.
Pflügers Arch (1977) 369, 251-257.
178. Schrader, J.
Sites of action and production of adenosine in the heart.
In: Purinergic receptors. G Burnstock (Ed.). Chapman & Hall, London (1981), pp 121-162.
179. Schrader, J, Schütz, W, Bardenheuer, H.
Role of s-adenosylhomocysteine hydrolase in adenosine metabolism in mammalian heart.
Biochem J (1981) 196, 65-70.

180. Schrader, J.
Metabolism of adenosine and sites of production in the heart.
In: Regulatory function of adenosine. RM Berne, TW Rall, R Rubio (Eds.), Martinus Nijhoff Publ, Boston (1983), pp 133-156.
181. Schütz, W, Schrader, J, Gerlach, E.
Different sites of adenosine formation in the heart.
Am J Physiol (1981) 240, H963-H970.
182. Schütz, W, Tuisl, E.
Evidence against adenylate cyclase-coupled adenosine receptors in the guinea pig heart.
Eur J Pharmacol (1981) 76, 285-288.
183. Schütz, W, Freissmuth, M.
Adenosine receptors in the heart: controversy about signal transmission.
Trend Pharmacol Sci (1985) 310-311.
184. Scott, JB, Daugherty, RM, Dabney, JM, Haddy, FJ.
Role of chemical factors in regulation of flow through kidney, hindlimb and heart.
Am J Physiol (1965) 208, 813-824.
185. Serruys, PW, De Jong, JW, Harmsen, E, Verdouw, PD, Hugenholtz, PG.
Effect of intracoronary nifedipine on high-energy phosphate metabolism during repeated pacing-induced angina and during experimental ischemia.
In: New therapy of ischaemic heart disease and hypertension. M. Kaltenbach and H.N. Neufeldt (Eds). Excerpta Medica, Amsterdam (1983), pp 340-353.
186. Shlafer, M, Kane, PF, Kirsh, MM.
Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischemic, reperfused heart.
J Thorac Cardiovasc Surg (1982) 83, 830-839.
187. Siaw, MFE, Mitchell, BS, Koller, CA, Coleman, MS, Hutton, JJ.
ATP depletion as a consequence of adenosine deaminase inhibition in man.
Proc Natl Acad Sci USA (1980) 77, 6157-6161.
188. Silverman, NA, Kohler, J, Feinberg, H, Levitsky, S.
Beneficial metabolic effect of nucleoside augmentation on reperfusion injury following cardioplegic arrest.
Chest (1983) 83, 787-792.
189. Smiseth, OA.
Inosine infusion in dogs with acute ischaemic left ventricular failure: favourable effects on myocardial performance and metabolism.
Cardiovasc Res (1983) 17, 192-199.
190. Soboll, S, Bünger, R.
Compartmentation of adenine nucleotides in the isolated working guinea pig heart stimulated by noradrenaline.
Hoppe-Seyler's Z Physiol Chem (1981) 362, 125-132.
191. Sollevi, A, Fredholm, BB.
The antilipolytic effect of endogenous and exogenous adenosine in canine adipose tissue in situ.
Acta Physiol Scand (1981) 113, 53-60.
192. Sollevi, A, Lagerkranser, M, Andreen, M, Irestedt, L.
Relationship between arterial and venous adenosine levels and vasodilatation during ATP- and adenosine-infusion in dogs.
Acta Physiol Scand (1984) 120, 171-176.
193. Somlo, E.
Adenosine triphosphate in paroxysmal tachycardia.
Lancet (1955) I, 1125.
194. Sparks, HV, Manfredi, JP, Phair, RD.
A compartmental model of changes in interstitial adenosine associated with increased myocardial adenosine.
In: Ca^{2+} entry blockers, adenosine, and neurohumors. GF Merrill, HR Weiss (Eds.), Urban & Schwarzenberg, Baltimore/Munich (1983), pp 189-205.

195. Sparks, HV, Bardenheuer, H.
Regulation of adenosine formation by the heart.
Circ Res (1986) 58, 193-201.
196. Stam, H, De Jong, JW.
Sephadex-induced reduction of coronary flow in the isolated rat heart: A model for ischemic heart disease.
J Mol Cell Cardiol (1977) 9, 633-650.
197. Starnes, JW, Wilson, DF, Erecinska, M.
Substrate dependence of metabolic state and coronary flow in perfused rat heart.
Am J Physiol (1985) 249, H799-H806.
198. Stewart, JR, Crute, SL, Loughlin, V, Hess, ML, Greenfield, LJ.
Allopurinol prevents free radical-mediated myocardial reperfusion injury.
Surg Forum (1983) 34, 325-327.
199. Swain, JL, Sabina, RL, McHale, PA, Greenfield, JC, Holmes, EW.
Prolonged myocardial nucleotide depletion after brief ischemia in the open-chest dog.
Am J Physiol (1982) 242, H818-H826.
200. Swain, JL, Sabina, RL, Peyton, RB, Jones, RN, Wechsler, AS, Holmes, EW.
Derangements in myocardial purine and pyrimidine nucleotide metabolism in patients with coronary artery disease and left ventricular hypertrophy.
Proc Natl Acad Sci USA (1982) 79, 655-659.
201. Taegtmeyer, H, Hems, R, Krebs, HA.
Utilization of energy-providing substrates in the isolated working rat heart.
Biochem J (1980) 186, 701-711.
202. Taegtmeyer, H, Roberts, AFC, Raine, AEG.
Energy metabolism in reperfused heart muscle: metabolic correlates to return of function.
J Am Coll Cardiol (1985) 6, 864-870.
203. Van Belle, H, Wynants, J, Goossens, F.
Formation and release of nucleosides in the ischemic myocardium. Is the guinea-pig the exception?
Basic Res Cardiol (1985) 80, 653-660.
204. Van der Vusse, GJ, Coumans, WA, Van der Veen, E, Drake, AJ, Flameng, W, Suy, R.
ATP, creatine phosphate and glycogen content in human myocardial biopsies: markers for the efficacy of cardioprotection during aorto-coronary bypass surgery.
Vasc Surg (1984) 18, 127-134.
205. Verhaege, RH, Vanhoutte, PM, Shepherd, JT.
Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenine nucleotides.
Circ Res (1977) 40, 208-215.
206. Vrobel, TR, Jorgensen, CR, Bache, RJ.
Myocardial lactate and adenosine metabolite production as indicators of exercise-induced myocardial ischemia in the dog.
Circulation (1982) 66, 555-561.
207. Wangler, RD, DeWitt, DF, Sparks, HV.
Effect of beta-adrenergic blockade on nucleoside release from the hypoperfused isolated heart.
Am J Physiol (1984) 247, H330-H336.
208. Ward, HB, St Cyr, JA, Cogordan, JA, Alyono, D, Bianco, RW, Kriett, JM, Foker, JE.
Recovery of adenine nucleotide levels after global myocardial ischemia in dogs.
Surgery (1984) 96, 248-255.

209. Watkinson, WP, Foley, DH, Rubio, R, Berne, RM.
Myocardial adenosine formation with increased cardiac performance in the dog.
Am J Physiol (1979) 236, H13-H21.
210. Watt, AH.
Sick sinus syndrome: an adenosine-mediated disease.
Lancet (1985) I, 786-788.
211. Watts, JA, Koch, CD, LaNoue, KF.
Effects of Ca^{2+} antagonism on energy metabolism: Ca^{2+} and heart function after ischemia.
Am J Physiol (1980) 238, H909-H916.
212. Wayne, EJ, Goodwin, JF, Stoner, HB.
The effect of adenosine triphosphate on the electrocardiogram of man and animals.
Br Heart J (1949) 11, 55-67.
213. Wedd, AM.
The action of adenosine and certain related compounds on the coronary flow of the perfused heart of the rabbit.
J Pharmacol Exp Ther (1931) 41, 355-366.
214. Wedd, AM, Drury, AN.
The action of certain nucleic acid derivatives on the coronary flow in the dog.
J Pharmacol Exp Ther (1934) 50, 157-164.
215. Wiedmeier, V, Spell, LH.
Effects of catecholamines, histamine and nitroglycerin on flow, oxygen utilization and adenosine production in the perfused guinea pig heart.
Circ Res (1977) 41, 503-508.
216. Williamson, JR.
Glycolytic control mechanisms. I. Inhibition of glycolysis by acetate and pyruvate in the isolated, perfused rat heart.
J Biol Chem (1965) 240, 2308-2321.
217. Winbury, MM, Papierski, DH, Hemmer, ML, Hambourger, WE.
Coronary dilator action of the adenine-ATP series.
J Pharmacol Exp Ther (1953) 109, 255-260.
218. Wolf, MM, Berne, RM.
Coronary vasodilator properties of purine and pyrimidine derivatives.
Circ Res (1956) 4, 343-348.
219. Woolliscroft, JO, Colfer, H, Fox IH.
Hyperuricemia in acute illness. A poor prognostic sign.
Am J Med (1982) 72, 58-62.
220. Zimmer, H-G, Ibel, H.
Ribose accelerates the repletion of the ATP pool during recovery from reversible ischemia of the rat myocardium.
J Mol Cell Cardiol (1984) 16, 863-866.

ENZYME ABBREVIATIONS AND NOMENCLATURE

Adenosine deaminase (ADA)	-Adenosine aminohydrolase (EC 3.5.4.4)
Adenosine kinase (AK)	-ATP: adenosine 5'-phosphotransferase (EC 2.7.1.20)
Adenylate kinase (AdK)	-ATP: AMP phosphotransferase (EC 2.7.4.3)
AMP-deaminase (AMP-D)	-AMP aminohydrolase (EC 3.5.4.6)
Creatine kinase (CK)	-ATP: creatine phosphotransferase (EC 2.7.3.2)
Hypoxanthine phosphoribosyl transferase (HPRT)	-IMP: pyrophosphate phosphoribosyltransferase (EC 2.4.3.8)
Nucleoside phosphorylase (NP)	-Purine-nucleoside: orthophosphate transferase (EC 2.4.2.1)
5'-Nucleotidase (5'NT)	-5'-Ribonucleotide phosphohydrolase (EC 3.1.3.5)
Xanthine oxidase (XO)	-Xanthine: oxygen oxidoreductase (EC 1.2.3.2)
Xanthine dehydrogenase (XD)	-Xanthine: NAD oxidoreductase (EC 1.2.1.37)
Adenosylhomocysteinase	-S-Adenosyl-L-homocysteine hydrolase (EC 3.3.1.1)

SAMENVATTING

Adenosine, een potente coronaire vaatverwijder, is een afbraakprodukt van het energierijke fosfaat ATP. ATP fungeert in het hart als de directe energiebron voor de contractie. Hoofdstuk 1 van dit proefschrift geeft een korte algemene inleiding over ATP-afbraak in relatie tot contractie, over het metabolisme dat de ATP-vorming verzorgt en over het mechanisme van adenosine vorming. De toepassingen, die de biochemie van de purines heeft voor cardiovasculair onderzoek, worden kort samengevat.

In Hoofdstuk 2 wordt de voornaamste literatuur samengevat, aangaande de rol van adenosine bij de regulatie van de coronaire vaatverwijding. Tevens worden de anticatecholaminerge effecten van adenosine besproken en de invloed van adenosine op de electromechanische activiteit van het hart (2.1-2.3).
Het metabolisme van adenosine, adenine nucleotiden en verwante purines wordt samengevat (2.4) met de nadruk op klinische toepassingen voor diagnostische en therapeutische doeleinden. Het potentieel gebruik van purines voor secundaire preventie in de cardiologische kliniek wordt besproken vanuit de experimentele aanwijzingen uit de literatuur.
Er zijn veel aanwijzingen dat er een directe relatie bestaat tussen de uitwas van adenosine en purines tijdens ischemie, het post-ischemisch ATP-gehalte en de functie van het hart (2.5).

Het experimentele gedeelte (Hoofdstuk 3 en de Appendix Papers) geeft meer gedetailleerde informatie over het purine metabolisme in geïsoleerde, geperfundeerde ratteharten onder normoxische en ischemische condities.
Wanneer adenosine in micromolaire concentratie wordt geïnfundeerd in geïsoleerde harten wordt een gedeelte (15-20%) opgenomen door het hart. Adenosine wordt echter voornamelijk gedeamineerd tot inosine, dat verder wordt afgebroken tot hypoxanthine, xanthine en uraat. Uraat is de voornaamste purineverbinding die uit het normoxische hart vrijkomt. De maximale activiteit, gemeten in hart homogenaten, van het enzym dat uraat vormt (xanthine oxydase/dehydrogenase) komt goed overeen met de maximale vorming van xanthine en uraat in geïsoleerde, ischemische harten. Een

gedeelte van dit enzym is aanwezig in de oxydase vorm en kan vrije radicalen vormen, die het hart irreversibel kunnen beschadigen. De in vitro eigenschappen van andere enzymen van het purine metabolisme (AMP-deaminase en S-adenosylhomocysteïne hydrolase) worden beschreven. Aanvullende perfusiestudies met specifieke remmers suggereren dat deze enzymen in belangrijke mate bijdragen aan de normoxische purinevorming (Appendix Papers II en IV). Er zijn een aantal aanwijzingen dat de purines die in het hart gevormd worden voor een deel afbraakprodukten zijn van IMP of GMP, en niet van AMP. Dit lijkt in het bijzonder zo te zijn tijdens normoxie.

Wanneer hypoxanthine of inosine aan het perfusaat van het hart worden toegevoegd, worden ze langzaam in de ATP-voorraad van het hart ingebouwd. Deze inbouw is sneller na een voorafgaande periode van ischemie en kan verder gestimuleerd worden door gelijktijdig infuus van ribose.

Er zijn aanwijzingen gevonden, dat versnelde adenosine vorming in het hart direct gerelateerd is aan de vorming van AMP (b.v. uit ATP). De oude hypothese dat adenosine constant met hoge snelheid geproduceerd wordt en onmiddellijk weer wordt gefosforyleerd tot AMP blijkt onrealistisch. De resultaten tonen aan dat de koppeling van adenosine- en purinevorming met de energiebalans van het hart sterker is dan werd aangenomen. Een geringe verstoring van de balans tussen ATP-vorming en ATP-afbraak zal een verhoogd vrijkomen van adenosine en ander purines veroorzaken en zal uiteindelijk tot een aanzienlijk ATP-verlies en verlies van functie leiden. Sommige van de gerapporteerde experimenten suggereren een benadering die deze vicieuze cirkel kan doorbreken, omdat ze de condities aangeven, waaronder versnelde ATP-synthese en functieherstel mogelijk zijn.

Nawoord

Allereerst wil ik mijn begeleider en promotor Jan Willem de Jong danken voor de door hem gegeven efficiënte "push", die projectgebonden (ZWO/FUNGO) onderzoek nodig heeft om langs een smalle weg vooruit te komen.
Prof. P.G. Hugenholtz dank ik voor de dynamische begeleiding bij de totstandkoming van dit proefschrift.
Eef Harmsen en Peter de Tombe bedank ik voor hun stimulerende enthousiasme, actieve steun en collegialiteit tijdens de eerste periode van het onderzoek. Selma Nieukoop, Liz Keijzer, Theo Verwoerd, Han Hegge, Ria Kanters-Stam, Tom Huizer, Bob Schoutsen, Petra Noomen, Sandra Lako, Rutger Stroeve, Mirjam Zweserijn, Peter van der Meer en John Tolboom vormden en vormen de collegiale background binnen het Cardiochemisch Lab. en gaven essentiële ondersteuning bij het eigenlijke werk, waarvoor ik hen veel dank verschuldigd ben.
Speciaal Selma Nieukoop en Ria Kanters-Stam hebben veel aan de vervaardiging van dit proefschrift bijgedragen.
Dank ook aan al diegenen binnen het Thoraxcentrum en de Medische Faculteit, die door "gewoon" hun werk te doen, hebben bijgedragen aan dit proefschrift.
Eamon Heskin en Ben Verwiel dank ik voor de door hen ingebrachte specifieke kennis.
De meeste dank ben ik echter verschuldigd aan iemand die liever niet genoemd wil worden voor de steun op belangrijke momenten en het stand houden op het thuisfront.
Ik draag dit proefschrift op aan mijn ouders, die mij altijd hebben gesteund en me hebben gestimuleerd om te gaan studeren.

CURRICULUM VITAE

10-02-1951	Geboren te Zeist.
1963 - 1969	Rijksscholengemeenschap "Schoonoord", Zeist. Eindexamen Gymnasium-B.
1969 - 1977	Rijks Universiteit Utrecht.
(1973)	Kandidaatsexamen scheikunde/propedeuse psychologie.
(1977)	Doctoraalexamen scheikunde met keuzerichting biochemie, bijvakken psychologie en organische chemie.
1978 - 1980	Nederlands Instituut voor Hersenonderzoek, Amsterdam. Vervangende Militaire Dienstplicht.
1980 - 1981	Rijks Universiteit Utrecht, Faculteit Diergeneeskunde, tijdelijk wetenschappelijk assistent.
1981 - heden	Erasmus Universiteit Rotterdam, Cardiochemisch Laboratorium (Thoraxcentrum), wetenschappelijk assistent, toegevoegd onderzoeker.

BIBLIOGRAPHY

Schoutsen, B, De Jong, JW, Harmsen, E, De Tombe, PP, Achterberg, PW.
Myocardial xanthine oxidase/dehydrogenase.
Biochim Biophys Acta (1983) 762, 519-524.

Harmsen, E, Verwoerd, TC, Achterberg, PW, De Jong, JW.
Regulation of porcine heart and skeletal muscle AMP-deaminase by adenylate energy charge.
Comp Biochem Physiol (1983) 75 B, 1-3.

Harmsen, E, De Tombe, PP, De Jong, JW, Achterberg, PW.
Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia.
Am J Physiol (1984) 246, H37-H43.

Achterberg, PW, De Tombe, PP, Harmsen, E, De Jong, JW.
Myocardial S-adenosylhomocysteine hydrolase is important for adenosine production during normoxia.
Biochim Biophys Acta (1985) 840, 393-400.

Achterberg, PW, Harmsen, E, De Jong, JW.
Adenosine deaminase inhibition and myocardial purine release during normoxia and ischemia.
Cardiovasc Res (1985) 21, 593-598.

Achterberg, PW, Stroeve, RJ, De Jong, JW.
Myocardial adenosine cycling rates during normoxia and under conditions of stimulated purine release.
Biochem J (1986) 235, 13-17.

Achterberg, PW, Harmsen, E, De Tombe, PP, De Jong, JW.
Balance of purine nucleotides and catabolites in the isolated ischemic rat heart.
Adv Exp Med Biol (1984) 165 B, 483-486.

Achterberg, PW, De Jong, JW.
Adenosine deaminase inhibition and myocardial adenosine metabolism during ischemia.
Adv Myocardiol (1985) 6, 465-472.

Harmsen, E, De Tombe, P, Achterberg, P, De Jong, JW.
Inosine incorporation into adenine- and guanine nucleotides in the isolated perfused rat heart is stimulated after ischemia.
Adv Exp Med Biol (1984) 165 B, 487-490.

Verwoerd, T, Harmsen, E, Achterberg, P, De Jong, JW.
Purification and characterization of pig heart AMP-deaminase.
Adv Exp Med Biol (1984) 165 B, 501-504.

Achterberg, PW, Bucx, JJJ, Nieukoop, AS, Schouten, VJA,
De Jong, JW, Ter Keurs, HEDJ.
High-energy phosphate metabolism in normoxic and ischemic
cardiac trabeculae and papillary muscles.
J Mol Cell Cardiol (submitted).

Achterberg, PW, Nieukoop, AS, Schoutsen, B, De Jong, JW.
Different purine metabolism in newborn heart coincides with
better ATP-preservation after ischemia and reperfusion.
Am J Physiol (submitted).

Huizer, T, De Jong, JW, Achterberg, PW.
Bepridil decreases ischemic high-energy phosphate breakdown in
heart.
J Cardiovasc Pharmacol (accepted for publication).

De Jong, JW, Achterberg, PW.
ATP stofwisseling.
In: Het Medisch Jaar. JA Bernards (Ed.). Bohn, Scheltema,
Holkema, Utrecht (1987), in press.

De Jong, JW, Achterberg, PW.
Developmental differences in myocardial ATP-metabolism.
Basic Res Cardiol (in press).

UITLEG VOOR DE LEEK

Het hart is een dikke spier die door samentrekken (kloppen) zorgt voor het pompen van zuurstofrijk bloed door het hele lichaam. De bloedvoorziening van de hartspier gaat via de kransslagaderen (coronairen). Het voortdurend kloppen van het hart vergt veel energie. Deze energie wordt in de spiercellen van het hart gevormd uit voedingsstoffen (suikers, vetzuren) die met behulp van zuurstof, dat via het coronaire bloed wordt aangevoerd, "verbrand" worden.
De "verbranding" waarvan hier sprake is, is eigenlijk een stapsgewijze omzetting van stoffen door middel van specifieke eiwitten (enzymen), die ieder een stapje van de afbraak voor hun rekening nemen (stofwisseling). De energie die vrijkomt, wordt vastgelegd in bepaalde "energierijke" stoffen (ATP en creatine fosfaat). Andere, vezelachtige, eiwitten die in de hartcel aanwezig zijn, kunnen dit ATP gebruiken om samen te trekken. Als alle cellen gelijktijdig samentrekken (contractie), klopt het hart. ATP is de "benzine" van het hart, die helaas niet rechtstreeks aan het hart toegediend kan worden, omdat ATP de celwand niet kan passeren.

Als het hart sneller gaat kloppen (stress, inspanning), wordt er tijdelijk zoveel ATP afgebroken, dat het zuurstofaanbod bij de normale doorstroming van de kransslagaders niet voldoende is. De afbraak van ATP is enige tijd sneller dan de aanmaak. Er kan dan een afbraakproduct van ATP uit het hart lekken (adenosine). Dit adenosine heeft een vaatverwijdende werking op de kransslagader, die daardoor meer bloed (en zuurstof) naar het hart kan voeren, zodat weer genoeg ATP gemaakt kan worden. Adenosine kan door verschillende eiwitten (enzymen) gemaakt worden. De afbraak van adenosine doorloopt op zijn beurt een aantal enzymatische stappen. Behalve adenosine lekken er bij zuurstoftekort dan ook een aantal andere afbraakprodukten van ATP (purines) uit het geïsoleerde rattehart, dat we als model gebruikt hebben. Door metingen van ATP en zijn afbraakprodukten ADP en AMP in het hartweefsel, en van adenosine en verdere afbraakprodukten in de vloeistof waarmee het hart wordt doorstroomd, hebben we een betere indruk gekregen van de regeling van de aanmaak en afbraak van adenosine in het hart. Uit onze experimenten kunnen we concluderen, dat de

vorming van adenosine zeer direct gekoppeld is aan het evenwicht tussen de aanmaak en afbraak van ATP. Een vroegere hypothese, die een constante, snelle vorming en directe afbraak van adenosine suggereerde, lijkt onjuist.

De door adenosine geregelde vaatverwijding in het hart is voornamelijk werkzaam in het min of meer "gezonde" hart, dat een verhoogde belasting te verduren heeft, bijvoorbeeld door stress, versnelde hartslag, verhoogde bloeddruk, licht zuurstoftekort. Een betere kennis van de stofwisseling van adenosine en ATP kan echter ook voor het "zieke" hart van belang zijn. Vernauwing van de kransslagader (atherosclerose, infarct) zal het zuurstofaanbod naar het hart sterk doen verminderen. Omdat dan meer ATP wordt afgebroken dan aangemaakt, zullen adenosine en zijn afbraak-produkten uit het hart verdwijnen. Zelfs bij een hersteld zuurstofaanbod zal het ATP-gehalte uiteindelijk lager zijn dan normaal. Omdat een verlaagd ATP-gehalte vaak gepaard gaat met een verlaagde hartfunktie, wordt er naar methodes gezocht om het ATP-gehalte weer snel op peil te brengen. Dit zou tevens een gedeeltelijke bescherming kunnen bieden tegen een volgende periode van slechte doorbloeding van het hart. Sommige van de in dit proefschrift beschreven experimenten laten zien dat versneld op peil brengen van de ATP-voorraad van het hart inderdaad mogelijk is.

APPENDIX PAPER

I

Myocardial xanthine oxidase/dehydrogenase

Biochimica et Biophysica Acta, 762 (1983) 519–524
Elsevier

BBA 11174

MYOCARDIAL XANTHINE OXIDASE/DEHYDROGENASE

BOB SCHOUTSEN, JAN WILLEM DE JONG, EEF HARMSEN, PETER P. DE TOMBE and PETER W. ACHTERBERG
Cardiochemical Laboratory, Thoraxcenter, Erasmus University Rotterdam, Postbus 1738, 3000 DR Rotterdam (The Netherlands)

(Received February 15th, 1983)

Key words: Adenosine catabolism; Xanthine oxidase; Xanthine dehydrogenase; Anoxia; Ischemia; HPLC; (Rat heart)

High-energy phosphates in heart muscle deprived of oxygen are rapidly broken down to purine nucleosides and oxypurines. We studied the role of xanthine oxidase/dehydrogenase (EC 1.2.3.2/EC 1.2.1.37) in this process with novel high-pressure liquid chromatographic techniques. Under various conditions, including ischemia and anoxia, the isolated perfused rat heart released adenosine, inosine and hypoxanthine, and also substantial amounts of xanthine and urate. Allopurinol, an inhibitor of xanthine oxidase, greatly enhanced the release of hypoxanthine. From the purine release we calculated that the rat heart contained about 18 mU xanthine oxidase per g wet weight. Subsequently, we measured a xanthine oxidase activity of 9 mU/g wet wt. in rat-heart homogenate. When endogenous low molecular weight inhibitors were removed by gel filtration, the activity increased to 31 mU/g wet wt. Rat myocardial xanthine oxidase seems to be present mainly in the dehydrogenase form, which upon storage at −20°C is converted to the oxidase form.

Introduction

During hypoxia, ATP in the heart is rapidly dephosphorylated, and adenosine and its catabolites are released (for review, see Ref. 1). Adenosine, inosine and hypoxanthine have been used as markers for ischemia in animals models [2–4] and in the clinical setting [5–7]. In these instances, hypoxanthine (and xanthine) were measured with an enzymological assay [8]. It had been assumed that hypoxanthine was the end-product of myocardial adenosine metabolism [9], but recently the release of urate from the isolated rat heart has been reported [10]. With a novel high-pressure liquid chromatographic (HPLC) assay we are now able to distinguish between hypoxanthine, xanthine and urate and measure them simultaneously [11]. Data on rat myocardial xanthine oxidase activity (EC 1.2.3.2) in vitro are conflicting: some investigators could not detect the enzyme [12,13] and others reported low activity [14,15].

Xanthine oxidase exists in mammals in two forms [16,17]: a dehydrogenase (EC 1.2.1.37), which uses NAD^+ as electron acceptor, and an oxidase, with O_2 as the electron acceptor [18]. Upon storage, the dehydrogenase form is converted to the oxidase form [18].

We assayed purine release from normoxic and ischemic/anoxic perfused rat heart and estimated the xanthine oxidase activity from the production of the oxypurines. Furthermore, we measured the activity of both enzyme forms in homogenates.

Part of this study have been published in abstract form [19,20].

Materials and Methods

Chemicals. All chemicals were analytical grade. Water was purified with the Milli-Ro4-Milli-Q system (Millipore, Bedford, MA, U.S.A.); pentobarbital was purchased from Abbot, Saint Rémy-sur-Avre, France; [8-^{14}C]xanthine (40–60 Ci/mol) was obtained from ICN, Irvine, CA, U.S.A.; bovine serum albumin and dithiothreitol were bought from

Sigma, St. Louis, MO, U.S.A.; allopurinol (4-hydroxypyrazolo(3,4-*d*) pyrimidine) was purchased from Burroughs-Wellcome, London, U.K.; the other purines were supplied by Boehringer Mannheim, F.R.G.

Heart perfusion. The experiments were performed with male Wistar rats (250–440 g), fed ad libitum. The animals were anesthetized with pentobarbital and the hearts were excised and arrested in ice-cold perfusion medium, a modified Tyrode solution containing glucose [21,22]. For our first set of experiments the working heart preparation [23] was used with a preload of 10 cmH_2O and afterload of 75 cmH_2O. For the induction of ischemia, a one-way ball valve was placed in the aortic outflow tract [23]. In a second series of experiments the Langendorff preparation was used [22,24] with 100 cmH_2O perfusion pressure. In this case hearts were made temporarily ischemic by reducing coronary flow with a roller pump [22], or anoxic by replacing oxygen in the perfusion liquid by nitrogen. The perfusion temperature was 37°C. Apex displacement was measured as described earlier [2].

Myocardial homogenate. After 10 min of retrograde perfusion [22] to remove blood, rat hearts were cooled in 30 mM K_2HPO_4/0.4 mM EDTA · Na_2 (pH 8.3) at 0°C, weighed and homogenized in 6 ml of this buffer at 0°C with a Virtis homogenizer (Virtis, Gardiner, NY, U.S.A.). Where indicated, the buffer contained 1 mM dithiothreitol, which prevents conversion of xanthine dehydrogenase to the oxidase form [25]. The homogenate was centrifuged at $1500 \times g$ for 10 min and the supernatant fluid was used immediately, or stored at −20°C. Just before the xanthine oxidase assay the homogenate was passed through a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) to remove low-molecular-weight compounds including endogenous inhibitors [14].

Xanthine oxidase / dehydrogenase assay. [8-^{14}C]Xanthine was purified by HPLC (see Ref. 11, but with 25 mM KH_2PO_4 (pH 6.0) as eluent). The assay conditions were 60 μM [8-^{14}C]xanthine (40 mCi/mol), 30 mM K_2HPO_4, 0.4 mM EDTA · Na_2 (pH 8.3), and heart homogenate diluted with 1% bovine serum albumin to 0.5 ml (cf. Ref. 26). The final volume was 1.0 ml. Incubation took place at 30°C for 60 min in the presence or absence of 0.1 mM NAD^+. The reaction was stopped with 0.2 ml 20% $HClO_4$. After centrifugation, the supernatant fluid was brought to pH 4–7 with 0.1 ml 1.7 M K_3PO_4, and the radioactive product and substrate were separated by HPLC (see Ref. 11, but with 50 mM KH_2PO_4 (pH 5.6)/3% v/v methanol). Flow rates up to 3 ml/min were used, and fractions of 1.5 ml were collected and counted with 10 ml Instagel (Packard Inst., Downers Grove, IL, U.S.A.) in a Packard B460C liquid scintillation counter. Urate was identified by the enzymatic peak shift method with urate oxidase (EC 1.7.3.3). One unit (U) of enzyme activity corresponds to the formation of 1 μmol product per min.

Purine assay. The purine nucleosides and oxypurines in perfusates were assayed with HPLC as has been described before [11,12].

Statistical analysis. We used Student's *t*-test and considered $P \leq 0.05$ (two-sided) as a significant difference. Results are given as mean values ± S.E. of the mean.

Results

Working heart

Coronary flow was 69 ± 4 ml/min per g dry wt. during the control perfusion (Table I). Hearts were made severely ischemic with a ball valve in the aortic outflow tract. At the end of the ischemic period, coronary flow had decreased by 90% ($P < 0.001$), and aortic flow had ceased. The concentration of purine nucleosides and oxypurines in the coronary effluent had increased significantly, but due to the low flow the measured purine release had decreased to less than half the original value. Coronary and aortic flow recovered only partially when the constriction was removed. 10 min post-ischemia, coronary flow was 35% of the control value ($P < 0.005$). It is noteworthy that these working hearts released substantial amounts of xanthine (Table I), which could indicate the presence of xanthine oxidase in rat heart.

Retrograde perfusion

In one set of experiments, coronary flow was 63 ± 5 ml/min per g dry wt. (Table II). In the effluents we also determined urate, which accounted for 74% of the purine nucleosides and oxypurines released during aerobic perfusion (Ta-

TABLE I

PRODUCTION OF PURINE NUCLEOSIDES AND OXYPURINES BY THE ISOLATED WORKING HEART

Hearts were isolated from F_1 hybrid rats from two inbred Wistar substrains. The perfused hearts were paced at 300 beats/min. At the end of 10-min periods of control perfusion, ischemia induced with a ball valve, or reperfusion, samples were taken for flow and purine measurements. Values are expressed (per g dry weight) as means ± S.E. ($n = 7$). CF, coronary flow; AoF, aortic flow; Ado, adenosine; Ino, inosine; Hyp, hypoxanthine; Xan, xanthine.

Condition	Flow (ml/min per g)		Production (nmol/min per g)			
	CF	AoF	Ado	Ino	Hyp	Xan
Control flow	69 ± 4	56 ± 12	0.1 ± 0.1	23 ± 9	16 ± 7	56 ± 20
Ischemia	7 ± 3 [a]	0 ± 0 [a]	3.6 ± 2.3	19 ± 9	5 ± 3	13 ± 7
Reperfusion	24 ± 11 [a]	9 ± 7 [a]	5.9 ± 2.0 [a]	61 ± 23 [a]	17 ± 6	32 ± 8

[a] $P \leq 0.05$ vs. control.

ble II). Ischemia, induced with a pump, caused a reduction in coronary flow of 78% ($P < 0.001$). Apex displacement, a measure for contractility, decreased 68% ($P < 0.001$). Purine release increased considerably: the release of adenosine, inosine and (hypo)xanthine increased at least 10-fold (Table II). However, urate production increased only 1.5-times, accounting for 18% of the purine nucleosides and oxypurines released during ischemia. During reperfusion, purine release decreased again. From the maximal release of xanthine and urate we calculated a rat-heart xanthine oxidase activity in situ of about 18 mU/g wet wt. at 37°C (during ischemia).

Subsequently, we perfused rat hearts with 0.1 mM allopurinol, an inhibitor of xanthine oxidase [27]. In this experiment coronary flow increased more than twice due to 10 min of anoxia in the presence or absence of allopurinol ($P < 0.001$). Under both conditions, the anoxia-induced decreases in apex displacement and in heart rate were 97% ($P < 0.001$) and 70–85% ($P < 0.02$), respectively. Hypoxanthine release increased from 3 ± 1 to 70 ± 11 nmol/min per g dry wt. due to anoxia ($P < 0.001$, Fig. 1). Reoxygenation caused the release to decrease to 13 ± 6 nmol/min per g dry wt. ($P < 0.001$ vs. anoxic value). We were unable to measure xanthine and urate in perfusates containing allopurinol due to interference of the drug in our HPLC assay. However, allopurinol augmented hypoxanthine release. After 10 min of normoxia, anoxia and reoxygenation the

TABLE II

PRODUCTION OF PURINE NUCLEOSIDES AND OXYPURINES BY THE ISOLATED NON-WORKING RAT HEART

Isolated perfused rat hearts were paced at 360 beat/min. After 20 min of aerobic perfusion, ischemia (induced with a roller pump) and reperfusion took place for 20 and 15 min, respectively. Measurements were made during the last 5 min of each period. Data are expressed as means ± S.E. with the number of experiments in parentheses. Coronary flow and purine release are expressed per g dry weight; apex displacement is given relative to the value after 5 min of aerobic perfusion. Ado, adenosine; Ino, inosine; Hyp, hypoxanthine; Xan, xanthine; UA, urate.

Condition	Coronary flow (ml/min per g)	Apex displacement (%)	Production (nmol/min per g)				
			Ado	Ino	Hyp	Xan	UA
Control flow (10)	63 ± 5	(100)	0.2 ± 0.2	4.2 ± 0.8	2.3 ± 0.5	1.1 ± 0.4	22 ± 5
Ischemia (6)	14 ± 1 [a]	32 ± 7 [a]	38 ± 19 [a]	83 ± 11 [a]	23 ± 6	17 ± 4 [a]	36 ± 2 [a]
Reperfusion (3)	47 ± 8 [b]	39 ± 20 [a]	6 ± 6	22 ± 18 [b]	5 ± 4	5 ± 3	29 ± 12

[a] $P < 0.05$ vs. control;
[b] $P < 0.05$ vs. ischemia.

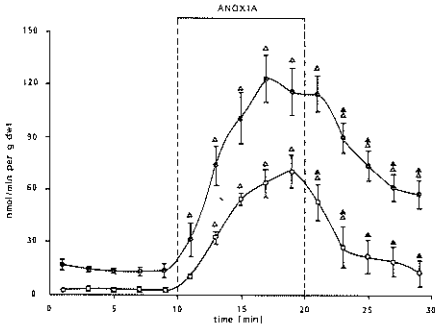


Fig. 1. Inhibition of hypoxanthine conversion by allopurinol in the retrogradely perfused heart. Hearts were isolated from F_1 hybrid rats from two inbred Wistar substrains. Hypoxanthine release in the presence (●), or absence (○) of 0.1 mM allopurinol is shown. Vertical bars denote S.E. ($n = 6$–7). (△) $P < 0.05$ vs. normoxia ($t = 9$ min), (▲) $P < 0.05$ vs. anoxia ($t = 19$ min). Hypoxanthine release in the presence of allopurinol was higher during normoxia, anoxia and reoxygenation ($P < 0.05$). dwt, dry weight.

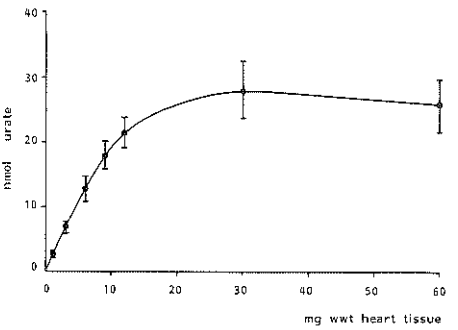


Fig. 2. Urate produced in 1 h by various amounts of rat heart xanthine oxidase/dehydrogenase. The activity was assayed with [8-^{14}C]xanthine, O_2 and NAD^+. Substrate and product were separated by HPLC and counted in a liquid scintillation counter. Data are means ± S.E. ($n = 3$). wwt, wet weight.

increase was 460% ($P < 0.005$, 64% ($P < 0.001$), and 420% ($P < 0.002$), respectively, of the value without the drug (Fig. 1). This indicates that, indeed, xanthine oxidase/dehydrogenase plays a role in the breakdown of hypoxanthine.

Xanthine oxidase / dehydrogenase in heart homogenate

The conversion of xanthine to urate by 12 mg fresh homogenate, which formed 6 nmol urate per h, was linear for 4 h. In our assay urate production by different amounts of heart tissue was linear until 20 nmol urate had been formed. The presence of xanthine dehydrogenase was demonstrated when NAD^+ was added to a fresh homogenate. The oxidase plus dehydrogenase activity was linear with urate production until 13 nmol urate had been formed in 1 h (Fig. 2).

The xanthine oxidase activity found in heart homogenate depended on the treatment of the preparation. Removal of small molecules by gel filtration of the homogenate caused an increase in xanthine oxidase activity from 9 to 31 mU/g wet wt. and from 10 to 27 mU/g wet wt. at 30°C for xanthine oxidase plus dehydrogenase activity. In a fresh homogenate, the xanthine oxidase activity was low, about 7 mU/g wet wt.; addition of NAD^+ stimulated 4-fold (Table III). Homogenization with dithiothreitol did not significantly influence the oxidase activity (Table III). After storage at −20°C, xanthine oxidase activity was high (31 mU/g wet wt.), and NAD^+-addition did not stimulate the activity (Table III). Allopurinol (0.1 mM) inhibited the xanthine oxidase and dehydrogenase activities by more than 98%.

TABLE III

XANTHINE OXIDASE AND DEHYDROGENASE ACTIVITIES IN RAT HEART HOMOGENATE

Perfused rat hearts were homogenized (with dithiothreitol where indicated) and centrifuged. The supernatant fluids were passed through a Sephadex G-25 column. With [8-^{14}C]xanthine at 30°C xanthine oxidase was assayed with O_2 (air) and xanthine oxidase/dehydrogenase with O_2 and NAD^+. The enzyme activities are given as means ± S.E. (n).

Preparation	Activity (mU/g wet wt.)	
	Xanthine oxidase	Xanthine oxidase plus xanthine dehydrogenase
Fresh	6.6 ± 0.4 (6) [a,b]	27.0 ± 0.8 (4)
Fresh + dithiothreitol	5.3 ± 0.5 (4) [a,b]	27.8 ± 1.4 (4)
Stored −20°C, 3 d	31.3 ± 2.8 (10)	33.1 ± 2.6 (3)

[a] $P < 0.001$ vs. stored;
[b] $P < 0.001$ vs. xanthine oxidase plus xanthine dehydrogenase.

Discussion

In a recent study on the degradation of ATP and its catabolites by the perfused rat heart, urate was found [10]. We observed that the anoxic and ischemic rat heart released substantial amounts of purine nucleosides and oxypurines including xanthine and urate. In the presence of allopurinol the release of hypoxanthine was much higher, so it seemed likely that xanthine oxidase was active in the isolated perfused rat heart, where it may play an essential role in the purine metabolism. Indeed, the direct assay of heart homogenates revealed xanthine oxidase activity, with a lower activity in fresh homogenates than in stored preparations. The difference could be accounted for by the presence of xanthine dehydrogenase activity. When NAD^+ was added, xanthine dehydrogenase activity could be demonstrated in fresh homogenates only. In the presence of NAD^+, urate production was inhibited during the course of the reaction. This is probably due to the accumulation of NADH which causes a feedback inhibition of xanthine dehydrogenase [28]. This mechanism may play a role in a purine salvage mechanism by reducing the breakdown of hypoxanthine [29] during ischemia when the concentration of this substrate for xanthine oxidase is high. The disappearance of NAD^+-stimulated urate production upon storage of heart tissue can be explained by the conversion of xanthine dehydrogenase to the oxidase form, as has been found for the liver enzyme [28]. In rat heart, Battelli et al. [16] could detect the dehydrogenase form only after treatment of the homogenate with dithioerythritol, while we found xanthine dehydrogenase activity in untreated homogenates. This different finding may be explained by assuming the presence of sulfhydryl oxidase in rat heart. The enzyme catalyzes the conversion of xanthine dehydrogenase to the oxidase form [30]. EDTA (present in our homogenization buffer) inhibits sulfhydryl oxidase [31]. Krenitsky et al. [17], who also used EDTA during homogenization, found xanthine dehydrogenase activities in many mammalian tissues without special treatment. Therefore, it seems important to include EDTA in the homogenization buffer if xanthine dehydrogenase activities are to be detected. However, in our study EDTA did not protect against conversion during storage at $-20°C$. Maybe this conversion is caused by a (slow) non-enzymatic oxidation of sulfhydryl groups by O_2.

Myocardial xanthine oxidase activity has also been reported [17,32,33] for cat, dog, sheep, cow, man and mouse. The presence of a dehydrogenase form and inhibition by endogenous small molecules, causing a low xanthine oxidase activity, may lead to false negative results as reported by Gandhi et al. [13] for rat heart. These problems may be avoided by using gel filtration [14] and NAD^+ or Methylene blue [15], an electron acceptor for both enzyme forms, giving a higher total activity [18]. When hearts are homogenized in the presence of EDTA and dithiothreitol, there still is xanthine oxidase activity. This may represent a xanthine oxidase with different catalytic properties compared to the dehydrogenase-derived oxidase, as has been found in mouse intestines [34].

We conclude that rat heart contains mainly xanthine dehydrogenase, which upon storage is converted to xanthine oxidase. The maximal activities calculated from xanthine and urate production in perfused rat hearts are comparable with the xanthine dehydrogenase values found in homogenates.

Acknowledgements

This work was supported in part by a grant from the Dutch Heart Foundation (grant number 80.041). We gratefully acknowledge the expert technical help of Ms. E. Keijzer in part of the experiments, and we thank Mrs. R. Kanters-Stam who helped with the preparation of this manuscript.

References

1 De Jong, J.W. (1979) in The Pathophysiology of Myocardial Perfusion (Schaper, W., ed.), pp. 719–750, Elsevier/North-Holland Biomedical Press, Amsterdam
2 Stam, H. and De Jong, J.W. (1977) J. Mol. Cell. Cardiol. 9, 633–650
3 De Jong, J.W., Verdouw, P.D. and Remme, W.J. (1977) J. Mol. Cell. Cardiol. 9, 297–312
4 Foley, D.H., Herlihy, J.T., Thompson, C.I., Rubio, R. and Berne, R.M. (1978) J. Mol. Cell. Cardiol. 10, 293–300
5 Fox, A.C., Reed, G.E., Glassmann, E., Kaltmann, A.J., and Silk, B.B. (1974) J. Clin. Invest. 53, 1447–1457

6 Remme, W.J., De Jong, J.W. and Verdouw, P.D. (1977) Am. J. Cardiol. 40, 55–62
7 Kugler, G. (1979) Eur. J. Cardiol. 9, 227–240
8 Olsson, R.A. (1970) Circ. Res. 26, 301–306
9 Berne, R.M., Rubio, R., Dobson, J.G., Jr. and Curnish, R.R. (1971) Circ. Res. 28 and 29, Suppl. I-115–I-119
10 Ronca-Testoni, S. and Borghini, F. (1982) J. Mol. Cell. Cardiol. 14, 177–180
11 Harmsen, E., De Jong, J.W. and Serruys, P.W. (1981) Clin. Chim. Acta 115, 73–84
12 Amory, N., Delbarre, F. and Auscher, C. (1978) C. R. Acad. Sci. Paris Ser. D 287, 1007–1009
13 Gandhi, M.P.S. and Ahuja, S.P. (1979) Zbl. Vet. Med. A. 26, 635–642
14 Ramboer, C.R.H. (1969) J. Lab. Clin. Med. 74, 828–835
15 Maguire, M.H., Lukas, M.C. and Rettie, J.F. (1972) Biochim. Biophys. Acta 262, 108–115
16 Battelli, M.G., Della Corte, E., Stirpe, F. (1972) Biochem. J. 126, 747–749
17 Krenitsky, T.A., Tuttle, J.V., Cattau, E.L., Jr. and Wang, P. (1974) Comp. Biochem. Physiol. 48B, 687–703
18 Stirpe, F. and Della Corte, E. (1969) J. Biol. Chem. 244, 3855–3863
19 De Jong, J.W., Harmsen, E. and Keijzer, E. (1980) J. Mol. Cell. Cardiol. 12, Suppl. 1, 33 (Abstr.)
20 Schoutsen, B., De Tombe, P.P., Harmsen, E., Keijzer, E. and De Jong, J.W. (1982) J. Clin. Chem. Biochem. 20, 416 (Abstr.)
21 Meyler, F.L., Bode, C. and Offerijns, F.G.J. (1958) Arch. Int. Physiol. Biochim. 66, 303–308
22 De Jong, J.W., Harmsen, E., De Tombe, P.P. and Keijzer, E. (1982) Eur. J. Pharmacol. 81, 89–96
23 Neely, J.R., Rovetto, M.J., Whitmer, J.T. and Morgan, H.E. (1973) Am. J. Physiol. 225, 651–658
24 Langendorff, O. (1895) Arch. Gesam. Physiol. 61, 291–332
25 Della Corte, E. and Stirpe, F. (1972) Biochem. J. 126, 739–745
26 Dougherty, T.M. (1976) Anal. Biochem. 74, 604–608
27 Elion, G.B., Callahan, S.W., Hitchings, G.H., Rundles, R.W. and Laszlo, J. (1962) Canc. Chemother. Rep. 16, 197–202
28 Della Corte, E. and Stirpe, F. (1970) Biochem. J. 117, 97–100
29 Kamiński, Z.W. and Jezewska, M.M. (1981) Biochem. J. 200, 597–603
30 Clare, D.A., Blakistone, B.A., Swaisgood, H.E. and Horton, H.R. (1981) 211, 44–47
31 Janolino, V.G. and Swaisgood, H.E. (1975) J. Biol. Chem. 250, 2532–2538
32 Al-Khalidi, U.A.S. and Chaglassian, T.H. (1965) Biochem. J. 97, 318–320
33 Lee, P.C. (1973) Arch. Biochem. Biophys. 157, 322–323
34 Krenitsky, T.A. and Tuttle, J.V. (1978) Arch. Biochem. Biophys. 185, 370–375

APPENDIX PAPER

II

Regulation of porcine heart and skeletal muscle AMP-deaminase by adenylate energy charge

Comp. Biochem. Physiol. Vol. 75B, No. 1, pp. 1 to 3, 1983
Printed in Great Britain.

0305-0491/83/050001-03$03.00/0

REGULATION OF PORCINE HEART AND SKELETAL MUSCLE AMP-DEAMINASE BY ADENYLATE ENERGY CHARGE

E. HARMSEN, T. C. VERWOERD, P. W. ACHTERBERG and J. W. DE JONG
Cardiochemical Laboratory, Thoraxcenter, Erasmus University Rotterdam, Rotterdam, The Netherlands

(*Received* 2 *September* 1982)

Abstract—1. Cytosol from pig skeletal muscle, but not heart, contains an inhibitor of AMP-deaminase (AMP-D, EC 3.5.4.6) which reduces AMP-D activity 8-fold.

2. Heart and skeletal muscle AMP-D have been purified to apparent homogeneity by cellulose phosphate and DEAE–Sephacel chromatography.

3. AMP-D from skeletal muscle is inhibited more severely than the heart enzyme by an increase in adenylate energy charge to levels exceeding 0.4. Nevertheless both enzymes seem to be regulated by the energy charge, which contrasts with reports for rabbit heart AMP-D.

INTRODUCTION

AMP-deaminase (AMP-D, EC 3.5.4.6) catalyzes the hydrolytic deamination of AMP to IMP and NH_3. The enzyme seems important in the regulation of the adenylate energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP) and the adenine nucleotide pool, together with 5'-nucleotidase (EC 3.1.3.5) and adenylate kinase (EC 2.7.4.3) (see review: De Jong, 1979). Relatively little is known about AMP-D in heart. In view of the presence of tissue specific isozymes of AMP-D (Ogasawara *et al.*, 1978; 1982), it seems likely that the physiological role of this enzyme may vary from tissue to tissue.

AMP-D has been purified to homogeneity from skeletal muscle of rabbit (Smiley *et al.*, 1967; Stankiewicz *et al.*, 1979), rat (Coffee & Kofke, 1975; Ranieri-Raggi and Raggi, 1976; Ogasawara *et al.*, 1978; Shiraki *et al.*, 1979; Stankiewicz *et al.*, 1979) and man (Stankiewicz, 1981; Ogasawara *et al.*, 1982). The heart enzyme has been purified only partially from a number of mammalian sources: rabbit (Chung & Bridger, 1976; Solano & Coffee, 1978; Barsacchi *et al.*, 1979), rat (Ogasawara *et al.*, 1975; Kaletha & Skladanowski, 1979), pig (Purzycka-Preis *et al.*, 1978), beef (Skladanowski *et al.*, 1981) and man (Kaletha *et al.*, 1979; Ogasawara *et al.*, 1982). Because of our interest in pig purine metabolism (De Jong & Goldstein, 1974; De Jong *et al.*, 1977) we decided to purify porcine heart and skeletal muscle AMP-D to homogeneity and studied the response of the enzymes to various adenylate energy charges*.

MATERIALS AND METHODS

Nucleotides

These were purchased from Boehringer, Mannheim, GFR.

AMP-D assay (see Smiley *et al.*, 1967)

The enzyme was incubated at 30°C with 50 mM imidazole.HCl, 20 mM sodium cacodylate, 0.5 M KCl, 1 mM 2-mercaptoethanol and 5 mM 5'-AMP (except for the experiments shown in Fig. 1). The pH was 6.5 and the final volume 1.0 ml. IMP formation was detected at 285 nm with a Gilford 2600 spectrophotometer (Gilford, Oberlin, OH).

Protein determination

The Coomassie Brilliant Blue (Biorad Laboratories, Munich, GFR) assay was used with bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Purification of AMP-D (see Coffee & Kofke, 1975; Purzycka-Preis *et al.*, 1978)

Each buffer contained 1 mM 2-mercaptoethanol. All steps were performed at 0–5°C. Heart or white skeletal muscle (100 g) from piglets (*Sus scrofa*, about 25 kg) was homogenized with 3 vol (v/w) of 0.18 M KCl–0.054 M KH_2PO_4–0.035 M K_2HPO_4 (pH 6.5). The homogenate was centrifuged for 30 min at 25,000 *g*, and the supernatant fluid passed through Nuova filter material (Hoffmann, Overath-Eulenthal, GFR). The eluate was stirred with 30 ml washed cellulose phosphate P11 (Whatman, Maidstone, UK; 5 g) for 1 hr. The slurry was then transferred to a glass column (1.5 × 12 cm) and washed with the extraction buffer until the effluent had an absorbance at 280 nm below 0.01. The enzyme was eluted with 1 M KCl (pH 6.5). Fractions with the highest specific activity were pooled and dialyzed against 0.045 M potassium phosphate (pH 7.2). The dialyzate was applied to a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden; 1.5 × 15 cm), which had been equilibrated with this phosphate buffer, and washed with 100 ml buffer. The enzyme was eluted with a linear gradient consisting of 100 ml each of 0.045 and 0.45 M potassium phosphate (pH 7.2).

Generation of energy charge ratios

AMP and ATP were incubated with adenylate kinase as described by Coffee & Solano (1977). The concentrations of AMP, ADP and ATP were determined by high performance liquid chromatography (Harmsen *et al.*, 1982). The individual nucleotide concentrations were found to be within 10% of the calculated ones.

* Part of this study has been presented at the 4th International Symposium on Human Purine and Pyrimidine Metabolism, Maastricht, The Netherlands (Verwoerd *et al.*, 1982).

C.B.P. 75/1B—A

Table 1. AMP-deaminase activity (U/mg protein) of pig heart and skeletal muscle cytosol

Treatment	Heart	Skeletal muscle
None	0.099 ± 0.006	0.340 ± 0.018*†
Gel filtration	0.104 ± 0.010	2.74 ± 0.038*

Mean values of 3 to 4 experiments ± SEM. Student's *t*-test: *P < 0.001 vs heart, †P < 0.001 vs treatment with Sephadex G-25 gel.

RESULTS

Activity

Table 1 shows that the activity of AMP-D in the cytosol of skeletal muscle could be increased 8-fold (to 2.7 U/mg protein) by removal of endogenous inhibitors. This treatment did not influence the heart enzyme, which had an activity of 0.10 U/mg protein.

Purification

In Table 2 data of the purification of AMP-D from heart and skeletal muscle are presented. With cellulose phosphate and DEAE–Sephacel chromatography they were purified 426 and 526 times, respectively. Disc electrophoresis on polyacrylamide (10% acrylamide, plus sodium dodecylsulfate) gave only one band. The cardiac enzyme did not show 5′-nucleotidase or adenosine deaminase (EC 3.5.4.4) activity. It was quite labile: 10% of enzymic activity disappeared when the preparation was kept overnight at −20°C. Repeated freeze-thawing caused the activity to disappear completely. Purified skeletal muscle AMP, on the other hand, remained active, when treated like this.

Properties of the purified enzymes

The apparent K_m of purified heart and skeletal muscle AMP-D was 3.8 and 1.2 mM, respectively. The Hill constant, n_H, was comparable for the two enzymes: 1.0 (heart AMP-D) and 1.1 (skeletal muscle AMP-D).

Effect of energy charge

Skeletal muscle AMP-D was inhibited by increasing adenylate energy charge (Fig. 1). Heart AMP-D showed a biphasic response. At energy charges which are probably physiological (>0.5), skeletal muscle AMP-D is inhibited 1.5–2.7 times more than heart AMP-D. The effects were most pronounced at lower (5 mM) total adenine nucleotide pool sizes.

DISCUSSION

The activity of AMP-D in pig skeletal muscle cytosol increased 8-fold, when low molecular weight components were removed (Table 1). It is of interest that Fishbein *et al.* (1981) found a specific inhibition of muscle AMP-D in serum and plasma of humans and animals. Apparently pig heart cytosol did not contain inhibitors which could be removed by gel filtration (Table 1). The V_{max} is 25 times lower than that in skeletal muscle cytosol (after gel filtration). Ogasawara *et al.* (1982) did a similar observation in human tissues.

Skeletal muscle AMP-D has been purified to homogeneity from a variety of species. However, the heart enzyme has only been completely purified from duck (Pekkel' and Kirkel', 1979), but not from the mammalian heart. Purzycka-Preis *et al.* (1978) obtained AMP-D from porcine heart by cellulose phosphate chromatography, but their preparation was presumably impure. We found it necessary to use DEAE–Sephacel chromatography after the cellulose phosphate step to obtain an apparently homogeneous preparation. Our procedure is considerably simpler than the one used by Pekkel' & Kirkel' (1979).

Purzycka-Preis *et al.* (1978) found a half-saturating substrate concentration for porcine heart AMP-D of 5 mM. This is comparable to our value (3.8 mM). For pig skeletal muscle AMP-D we found the K_m to be 3-fold lower. This contrasts findings by Ogasawara *et al.* (1975) who reported that rat skeletal muscle AMP-D has a K_m which is 3–4 times higher than that of heart.

Purzycka-Preis *et al.* (1978) reported cooperative binding of AMP for pig heart AMP-D ($n_H = 2.35$). This value decreased to about 1 on addition of ATP and liposomes. In our hands $n_H = 0.96$ was found for the purified enzyme without any addition, which is similar to the value reported for rat heart AMP-D (Kaletha & Skladanowski, 1979). We conclude from our studies that the number of binding-sites for AMP on AMP-D from pig heart and skeletal muscle is the same.

Solano & Coffee (1978) reported that rabbit heart AMP-D was unaffected by energy charges below 0.9, in contrast to the skeletal muscle enzyme which increased gradually in activity when the energy charge was decreased from 1.0 to 0. We observed that pig skeletal muscle enzyme AMP-D behaved comparably to that from rabbit (Fig. 1). Although the inhibition observed with physiological energy charges was less for the heart enzyme (Fig. 1), AMP-D from porcine heart seems to be regulated by the energy charge, whereas rabbit heart AMP-D always seems to be in

Table 2. Purification of pig heart and skeletal muscle AMP-deaminase

Purification step	Specific activity (U/mg protein)		Purification (×)		Yield (%)	
	Heart	SM	Heart	SM	Heart	SM
Supernatant fluid	0.10	0.30	(1)	(1)	(100)	(100)
Cellulose phosphate	20	75	208	252	20	62
DEAE-Sephacel	30	156	426	526	11	42

SM, skeletal muscle.

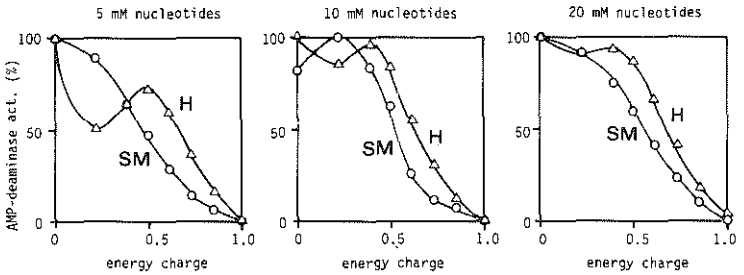


Fig. 1. Response of AMP-D to variation in the adenylate energy charge at different total adenine nucleotide pool sizes. Purified enzyme of pig skeletal muscle (SM, 3 μg) and heart (H, 10 μg) were tested in triplicate. Maximal activities (μmol/min) observed at 5 mM nucleotides: 0.39 (SM) and 0.12 (H); at 10 mM nucleotides: 0.53 (SM) and 0.30 (H); at 20 mM nucleotides: 0.49 (SM) and 0.38 (H).

the activated state (Solano & Coffee, 1978; Barsacchi *et al.*, 1979).

REFERENCES

Barsacchi R., Ranieri-Raggi M., Bergamini C. & Raggi A. (1979) Adenylate metabolism in the heart. Regulatory properties of rabbit cardiac adenylate deaminase. *Biochem. J.* **182**, 361–366.

Chung L. & Bridger W. A. (1976) Activation of rabbit cardiac AMP aminohydrolase by ADP: a component of a mechanism guarding against ATP depletion. *FEBS Lett.* **64**, 338–340.

Coffee C. J. & Kofke W. A. (1975) Rat muscle 5'-adenylic acid aminohydrolase. I. Purification and subunit structure. *J. biol. Chem.* **250**, 6653–6658.

Coffee C. J. & Solano C. (1977) Rat muscle 5'-adenylic acid aminohydrolase. Role of K^+ and adenylate energy charge in expression of kinetic and regulatory properties. *J. biol. Chem.* **252**, 1606–1612.

De Jong J. W. (1979) Biochemistry of acutely ischemic myocardium. In *The Pathophysiology of Myocardial Perfusion* (Edited by Schaper W.), pp. 719–750. Elsevier North-Holland Biomedical Press, Amsterdam.

De Jong J. W. & Goldstein S. (1974) Changes in coronary venous inosine concentration and myocardial wall thickening during regional ischemia in the pig. *Circulation Res.* **35**, 111–116.

De Jong J. W., Verdouw P. D. & Remme W. J. (1977) Myocardial nucleoside and carbohydrate metabolism and hemodynamics during partial occlusion and reperfusion of pig coronary artery. *J. molec. Cell. Cardiol.* **9**, 297–312.

Fishbein W. N., Davis J. I., Nagarajan K. & Smith M. J. (1981) Specific serum/plasma inhibitor of muscle adenylate deaminase. *ICRS Med. Sci.-Biochem.* **9**, 178–179.

Harmsen E., De Tombe P. Ph. & De Jong J. W. (1982) Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography. *J. Chromat.* **230**, 131–136.

Kaletha K. & Skladanowski A. (1979) Regulatory properties of rat heart AMP deaminase. *Biochim. biophys. Acta* **568**, 80–90.

Kaletha K., Skladanowski A., Bogdanowicz S. & Żydowo M. (1979) Purification and some regulatory properties of human heart adenylate deaminase. *Int. J. Biochem.* **10**, 925–929.

Ogasawara N., Goto H. & Watanabe T. (1975) Isozymes of rat AMP deaminase. *Biochim. biophys. Acta* **403**, 530–537.

Ogasawara N., Goto H., Yamada Y. & Watanabe T. (1978) Distribution of AMP-deaminase isozymes in rat tissues. *Eur. J. Biochem.* **87**, 297–304.

Ogasawara N., Goto H., Yamada Y., Watanabe T. & Asano T. (1982) AMP deaminase isozymes in human tissues. *Biochim. biophys. Acta* **714**, 298–306.

Pekkel' V. A. & Kirkel' A. Z. (1979) Purification and certain physicochemical properties of myocardial adenylate deaminase. *Biokhimiya (Engl. Transl.)* **44**, 1311–1319.

Purzycka-Preis J., Prus E., Woźniak M. & Żydowo M. (1978) Modification by liposomes of the adenosine triphosphate-activating effect on adenylate deaminase from pig heart. *Biochem. J.* **175**, 607–612.

Ranieri-Raggi M. & Raggi A. (1976) Specific modification of the GTP binding sites of rat 5'-adenylic acid aminohydrolase by periodate-oxidized GTP. *Biochim. biophys. Acta* **445**, 223–233.

Shiraki H., Ogawa H., Matsuda Y. & Nakagawa H. (1979) Interaction of rat muscle AMP deaminase with myosin. I. Biochemical study of the AMP deaminase and myosin in rat muscle. *Biochim. biophys. Acta* **566**, 335–344.

Skladanowski A., Kaletha K. & Żydowo M. (1981) Hydro- and thermodynamic properties of bovine heart AMP-deaminase. *Int. J. Biochem.* **13**, 865–869.

Smiley K. L., Berry A. J. & Suelter C. H. (1967) An improved purification, crystallization, and some properties of rabbit muscle 5'-adenylic acid deaminase. *J. biol. Chem.* **242**, 2502–2506.

Solano C. & Coffee C. J. (1978) Differential response of AMP deaminase isozymes to changes in the adenylate energy charge. *Biochem. biophys. Res. Commun.* **85**, 564–571.

Stankiewicz A. (1981) AMP-deaminase from human skeletal muscle. Subunit structure, amino-acid composition and metal content of the homogeneous enzyme. *Int. J. Biochem.* **13**, 1177–1183.

Stankiewicz A., Spychala J., Skladanowski A. & Żydowo M. (1979) Comparative studies on muscle AMP-deaminase—I. Purification, molecular weight, subunit structure and metal content of the enzymes from rat, rabbit, hen, frog and pikeperch. *Comp. Biochem. Physiol.* **62B**, 363–369.

Verwoerd T. C., Harmsen E., Achterberg P. W., & De Jong J. W. (1982) Purification and characterization of pig heart AMP-deaminase. *J. clin. Chem. Biochem.* **20**, 431 (Abstr.)

APPENDIX PAPER

III

Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia

Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia

EEF HARMSEN, PETER P. DE TOMBE, JAN WILLEM DE JONG, AND PETER W. ACHTERBERG
Cardiochemical Laboratory (Thoraxcenter), Erasmus University, 3000 DR Rotterdam, The Netherlands

HARMSEN, EEF, PETER P. DE TOMBE, JAN WILLEM DE JONG, AND PETER W. ACHTERBERG. *Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia.* Am. J. Physiol. 246 (Heart Circ. Physiol. 15): H37–H43, 1984.—Increasing therapeutic use is made of purines for the treatment of ischemic heart disease, but little is known about regulatory mechanisms involved. Therefore we perfused isolated rat hearts with 0.02 mmol/l [8-^{14}C]hypoxanthine or inosine. Under normoxic conditions about 1% is taken up by the heart and partially used for synthesis of ATP and GTP at a rate of 0.4 and 0.1 nmol·min^{-1}·g dry wt^{-1}, respectively. After relatively mild ischemia (coronary flow reduction of 70% for 20 min), no increase in myocardial purine uptake is observed, but ATP and GTP synthesis rates are doubled ($P < 0.001$). D-Ribose stimulates the hypoxanthine incorporation rate in normoxic perfused rat hearts to 1.1 and 0.5 nmol·min^{-1}·g dry wt^{-1} for ATP and GTP, respectively, which is further increased during postischemic perfusion. About 80% of the [8-^{14}C]inosine or [8-^{14}C]hypoxanthine passes through the heart unchanged, while 15% is converted to (hypo)xanthine and uric acid. We conclude from these experiments that inosine and hypoxanthine incorporation into ATP and GTP is at least partly regulated by the availability of 5-phosphoribosyl-1-pyrophosphate.

nucleotide biosynthesis; salvage; oxypurine; nucleoside; ribose; heart; rat

IN THE AEROBIC HEART a critical balance exists between production of ATP and its utilization. If the heart becomes ischemic, this delicate balance is disturbed, and energy-rich phosphates are broken down to the nucleosides and purine bases adenosine, inosine, hypoxanthine, xanthine, and uric acid. These apolar compounds can pass the cellular membrane and enter the bloodstream (2, 9). Because of this loss of purines, ATP levels will remain below control levels after reoxygenation (see, e.g., Ref. 18). To restore these adenine nucleotide pools three main pathways exist (Fig. 1). *1*) In adenosine phosphorylation, adenosine is directly phosphorylated to AMP by adenosine kinase. The maximal incorporation rate in rat heart amounts to about 50 nmol·min^{-1}·g dry wt^{-1} (16). This pathway is energetically economical. However, the adenosine concentration in blood is low, and this could be a restricting factor for adenosine phosphorylation. *2*) In hypoxanthine salvage, hypoxanthine can be phosphorylated with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form IMP (5, 12, 14, 22). IMP is a crucial branching point between adenine and guanine nucleotide synthesis. In rat heart the hypoxanthine incorporation rate into ATP can amount to 3 nmol·min^{-1}·g dry wt^{-1} (14). *3*) In de novo synthesis, purine nucleotides (adenine and guanine nucleotides) are also synthesized from small precursor molecules (among others, glycine, CO_2). In a 10-step synthesis from PRPP, IMP is formed. De novo production rate of IMP in rat heart is about 0.1 nmol·min^{-1}·g dry wt^{-1} (24). This process is stimulated after anoxia, ischemia, and hypertrophy. As an explanation for this phenomenon, an enhanced synthesis of PRPP by acceleration of the hexose-monophosphate shunt has been proposed (24). To bypass this shunt, ribose (a precursor of PRPP) can also be supplied (25).

We decided to study the incorporation of [8-^{14}C]hypoxanthine into normoxic or postischemic rat hearts in the presence of D-ribose to investigate whether hypoxanthine incorporation is also PRPP dependent. Because inosine, a precursor of hypoxanthine, is used as a cardioprotective and vasodilatory agent (4, 10, 11, 21, 23), [8-^{14}C]inosine incorporation rates in aerobic and postischemic rat hearts were also determined. Mammalian cells lack inosine kinase (22); therefore inosine is converted to hypoxanthine and ribose-1-phosphate. Subsequently, part of the hypoxanthine is converted to IMP; ribose-1-phosphate can serve as a precursor of PRPP.

Hypoxanthine and inosine are incorporated not only into adenine nucleotides but also into guanine nucleotides. To our knowledge no data about purine incorporation into myocardial guanine nucleotides have been published, although Swain et al. (20) recently investigated the incorporation of 5-aminoimidazol-4-carboxamide riboside (AICAR, an intermediate of the de novo pathway) in dog heart and found repletion of ATP and GTP levels.

MATERIALS AND METHODS

Perfusion protocol. Male Wistar rats [315 ± 34 (SD) g] were used. Hearts were removed and perfused as described earlier (3). The basic perfusion buffer (pH 7.4) consisted of (in mM) NaCl 125, KCl 4.7, $CaCl_2$ 1.35, $NaHCO_3$ 20, NaH_2PO_4 0.4, $MgCl_2$ 1.0, and D-glucose 10 and was equilibrated with 95% O_2-5% CO_2. The perfusion pressure was 72 mmHg, measured with a Statham pressure transducer (model P2306, Gould, Oxnard, CA). The hearts were paced at 300 beats/min, and the temperature

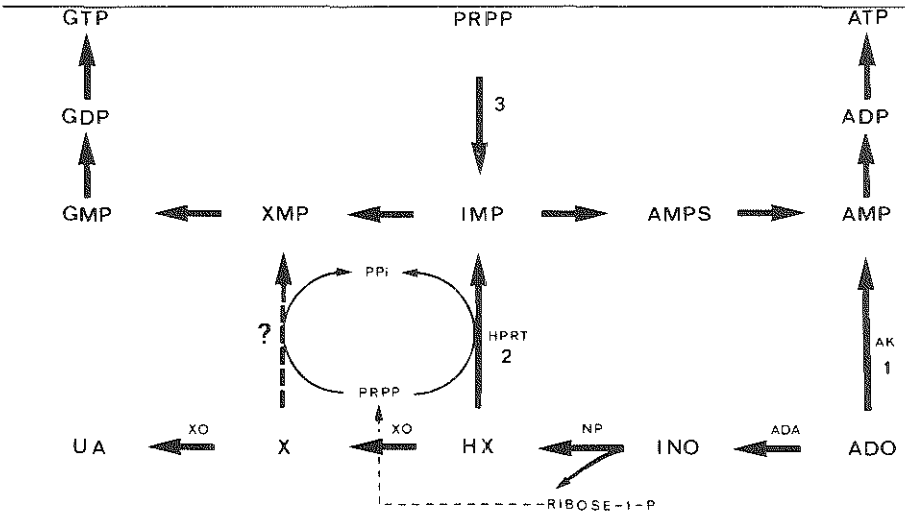


FIG. 1. Biosynthesis of ATP and GTP. *Pathway 1*, adenosine phosphorylation; *pathway 2*, hypoxanthine salvage; *pathway 3*, de novo synthesis. ADA, adenosine deaminase (EC 3.5.4.4); ADO, adenosine; AK, adenosine kinase (EC 2.7.1.20). AMPS, adenylosuccinate; HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); INO, inosine; NP, purine-nucleoside phosphorylase (EC 2.4.2.1); PP_i, pyrophosphate; PRPP, phosphoribosyl pyrophosphate; UA, uric acid; XAN, xanthine; XMP, xanthine-5′-monophosphate; XO, xanthine oxidase (EC 1.2.3.2).

was 37.0 ± 0.5°C.

The hearts were equilibrated with the buffer for 20 min (*period 1*: aerobic perfusion). Then global ischemia was induced with a roller pump at a fixed flow of 2.5 ml/min (flow reduction by 65–73%) for 20 min (*period 2*: ischemia), followed by 5 min of reperfusion (*period 3*: reactive hyperemia). Hereafter perfusion took place for an additional 15 min (*period 4*: reperfusion) with 0.02 mM [8-^{14}C]hypoxanthine, 0.02 mM [8-^{14}C]inosine, or 0.02 mM [8-^{14}C]hypoxanthine + 0.5 mM ribose (all dissolved in the perfusion fluid, radioactivity 5 μCi/l). After *period 4*, hearts were perfused with basic perfusion medium for 1 min to wash out the labeled substrates from the blood vessels. In the control group no ischemia was induced during *period 2*.

Chemicals. All chemicals were analytic grade. Water was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA). [8-^{14}C]hypoxanthine and [8-^{14}C]inosine were supplied by Radiochemical Centre (Amersham, Bucks., UK; sp act 50–60 Ci/mol, diluted to 0.25 Ci/mol before use).

Analytic methods. Samples were collected and stored on ice until analysis. No changes in purine composition of the perfusates were seen within the storage period, which was 12 h at the most. Adenosine(catabolites) were assayed by a slightly modified version of the high-performance liquid chromatography (HPLC) method described by Harmsen et al. (6). A μBondapak C_{18} column (Waters Associates, Milford, MA) was eluted at a flow rate of 3.0 ml/min with 75 mM KH_2PO_4, pH 4.50, to which methanol (30 ml/l) had been added. Two hundred microliters of the coronary effluent were injected on the system. Peaks were integrated (Varian CDS-111, Varian, Palo Alto, CA) and compared with standards. From radioactive samples the various HPLC peaks were collected. Ten milliliters of Instagel (Packard Instruments, Downers Grove, IL) were added, and radioactivity was determined with a Packard Tricarb 2650 liquid scintillation counter.

The perfusion experiment was terminated by freeze clamping the heart between two aluminum blocks precooled in liquid N_2 (−190°C). After being weighed, the heart was ground in a mortar, and about 0.5 g was mixed with 3.0 ml 0.8 N $HClO_4$ at −190°C. The other half was freeze dried to determine dry weight. The acid homogenate was thawed and centrifuged at 4°C. Two milliliters of the supernatant fluid were neutralized with about 200 μl 6 N KOH. After centrifugation, 20 μl of the supernatant fluid were used for the determination of nucleotides and creatine phosphate with HPLC according to Harmsen et al. (8).

To determine ^{14}C radioactivity in the ATP and GTP fractions, a fast, isocratic HPLC separation was developed. A Partisil-10-SAX column (Whatman, Maidstone, UK) was eluted at 3.0 ml/min with 0.42 M KH_2PO_4, pH 4.20. One milliliter of neutralized heart extract was injected. Fractions were collected and counted (Fig. 2). In addition 50 μl of this extract were counted to determine total ^{14}C-radioactivity uptake in the heart. Values were corrected for blanks. ^{14}C-counting efficiency was 85–90%.

Coronary flow measurements. Mean coronary flow was measured by timed collection of perfusate in the periods described above.

Statistical analysis. For statistical evaluation, analysis

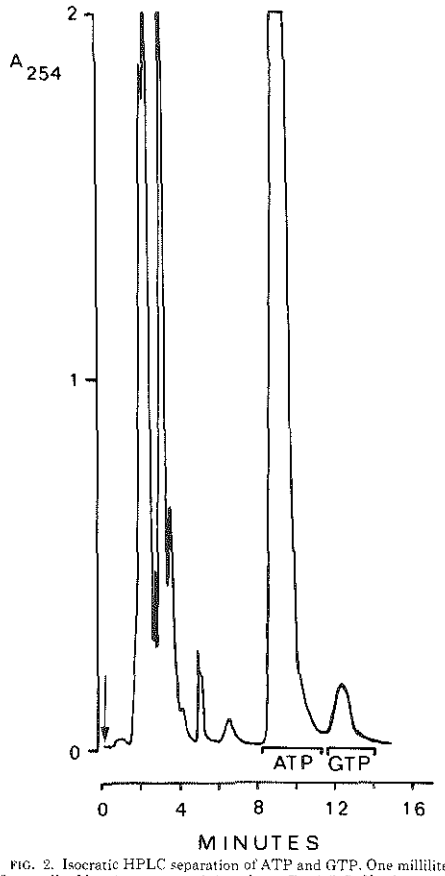


FIG. 2. Isocratic HPLC separation of ATP and GTP. One milliliter of neutralized heart extract was injected on a Partisil-SAX column and eluted with a flow of 3.0 ml/min with 0.42 mM KH_2PO_4, pH 4.20.

of variance was used (2-way classification); further comparisons were made using Scheffé's method for multiple comparisons (19). When only two means were compared, Student's unpaired t test was used. $P > 0.05$ was considered not significant (NS). Results are given as means ± SE. Three treatments (hypoxanthine, inosine, or hypoxanthine + ribose) were tested during two conditions (normoxia or reperfusion). Analysis of variance revealed that one experiment (hypoxanthine + ribose during normoxia), in which the ATP and GTP biosynthesis rates were 4.43 and 0.83 nmol·min^{-1}·g dry wt^{-1}, respectively, contributed the major part of variance. The means ± SD for all experiments ($n = 26$) were 1.06 ± 0.71 and 0.31 ± 0.11 nmol·min^{-1}·g dry wt^{-1}, respectively. According to the rejection rule of Anscombe and Tukey (see Ref. 19) with a premium of 5%, the values of the experiment mentioned earlier were considered as gross errors and rejected. The recalculated biosynthesis rates for ATP and GTP for all experiments were 0.92 ± 0.19 and 0.29 ± 0.06 nmol·min^{-1}·g dry wt^{-1}, respectively ($n = 25$).

RESULTS

In Fig. 3 mean coronary flows during the various periods are presented. During *period 2*, flow was restricted to 13 ml·min^{-1}·g dry wt^{-1} (flow reduction of 80%, $P < 0.001$ vs. control). In the first 5 min of reperfusion the average flow increased to above control level (*level 3*, reactive hyperemia; $P < 0.001$ vs. control), but in *period 4* flows in control and postischemic hearts were comparable. In preliminary experiments we investigated the time dependency of hypoxanthine and inosine release during and after myocardial ischemia (Fig. 4). After induction of ischemia, inosine and hypoxanthine appeared in the coronary effluent. However, within the first 5 min of reperfusion (reactive hyperemia, *period 3*) inosine and hypoxanthine returned to control levels (<0.1 μM).

Total normoxic purine release (i.e., adenosine, inosine, hypoxanthine, xanthine, and uric acid) was about 20 nmol·min^{-1}·g dry wt^{-1} (Fig. 5; *period 2*, control). This purine release was increased about six times during ischemia ($P < 0.001$ vs. control) and nine times during reactive hyperemia ($P < 0.001$ vs. control). During normoxic perfusion 64% of the purines released consisted of uric acid, but during ischemia this percentage fell to 28%. The percentages of the other purine compounds in the perfusate collected during normoxia were (with ischemic values in parentheses) adenosine 5 (11), inosine 17 (38), hypoxanthine 8 (13), and xanthine 5 (9), respectively.

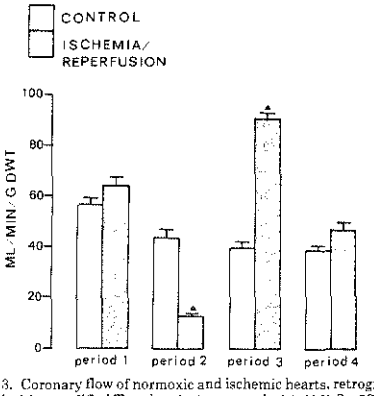


FIG. 3. Coronary flow of normoxic and ischemic hearts, retrogradely perfused with a modified Tyrode solution, gassed with 95% O_2–5% CO_2, at a perfusion pressure of 72 mmHg and a heart rate of 300 beats/min. Ischemia was introduced with a roller pump at a fixed flow of 2.5 ml/min. *Period 1*, aerobic perfusion (0–20 min); *period 2*, ischemic period (20–40 min); *period 3*, reactive hyperemia (40–45 min); and *period 4*, reperfusion (45–60 min). In control hearts no flow reduction was used. Values are means ± SE ($n = 4$–6). ▲, $P < 0.001$ vs. control (t statistics).

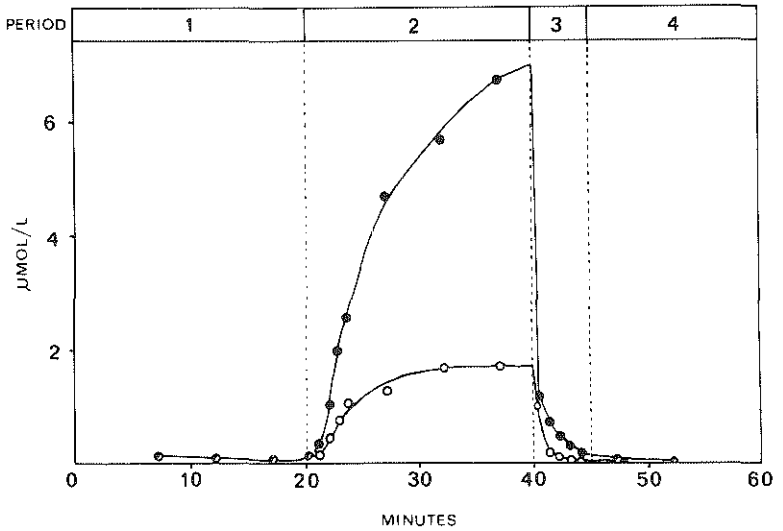


FIG. 4. Myocardial release of hypoxanthine (○—○) and inosine (●—●). Both compounds were determined in effluent with HPLC (see METHODS). *Periods 1–4* are defined in legend to Fig. 3. Average of at least 3 experiments.

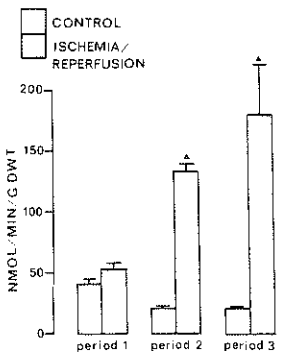


FIG. 5. Total purine release (adenosine + inosine + hypoxanthine + xanthine + uric acid) from isolated rat hearts. *Periods 1–3* are defined in legend of Fig. 3. Values are means ± SE (n = 4–6). ▲, $P <$ 0.001 vs. control (t statistics).

TABLE 1. *Uptake and incorporation rates of hypoxanthine and inosine*

Addition	Total Uptake	ATP	GTP
		Normoxia	
Hypoxanthine	84 ± 12 (100%)	6.4 ± 0.9 (8%)	2.0 ± 0.4 (2%)
Hypoxanthine + ribose	93 ± 21 (100%)	16.0 ± 1.3 (18%)	5.3 ± 0.4 (7%)
Inosine	107 ± 8 (100%)	7.3 ± 1.3 (7%)	2.9 ± 0.4 (3%)
		Reperfusion	
Hypoxanthine	96 ± 7 (100%)	14.6 ± 0.5 (15%)	3.9 ± 0.3 (4%)
Hypoxanthine + ribose	91 ± 14 (100%)	22.3 ± 1.3 (26%)	7.2 ± 0.7 (9%)
Inosine	123 ± 14 (100%)	16.3 ± 2.5 (14%)	4.9 ± 0.6 (4%)

Values are means ± SE in nmol·15 min^{-1}·g dry wt^{-1} (n = 3–6). Figures in parentheses are percents of total ^{14}C-radiolabel uptake by tissue. [8-^{14}C]hypoxanthine or [8-^{14}C]inosine conversion to ATP and GTP and uptake of radioactivity were determined in acid extracts of freeze-clamped hearts. For statistical analysis, see text.

In Table 1, the biosynthesis of ATP and GTP from 0.02 mM hypoxanthine or inosine is presented. Analysis of variance for hypoxanthine and inosine incorporation rates in the ATP fraction showed no significant interaction (P = 0.65) between the conditions normoxia and reperfusion on the one hand and the three treatments (infusion of hypoxanthine, of hypoxanthine + ribose, and of inosine) on the other hand. Thus the pattern for the three treatments was similar for normoxia compared with reperfusion. Incorporation rates during reperfusion were significantly ($P < 0.0001$) increased compared with the normoxic controls. Comparison of the three treatments proved them to be statistically different ($P <$ 0.0001). In addition, by Scheffé's method of multiple comparison the incorporation rates in the group treated with hypoxanthine + ribose were found to be different from the groups treated with hypoxanthine or inosine, the latter two not being different. Also the GTP incorporation rates showed no significant interaction (P = 0.97) between normoxia/reperfusion and the three treatments. The significant differences paralleled those of the ATP incorporation rates.

Besides incorporation into nucleotides, the other met-

TABLE 2. *Metabolic fate of [8-^{14}C]hypoxanthine and [8-^{14}C]inosine*

Addition	Percent of Label Infused					
	Myocardial uptake	Adenosine	Inosine	Hypoxanthine	Xanthine	Uric acid
			Normoxia			
Hypoxanthine	0.9 ±0.2	0	0	82.7 ±0.7	5.5 ±0.2	2.7 ±0.6
Hypoxanthine + ribose	0.9 ±0.1	0	0.5 ±0.2	83.8 ±2.9	5.7 ±0.8	4.5 ±0.4
Inosine	1.4 ±0.1	0	81.1 ±2.9	3.8 ±0.2	2.4 ±0.2	7.3 ±0.1
			Reperfusion			
Hypoxanthine	0.8 ±0.1	0	0.2 ±0.1	83.1 ±0.6	6.1 ±0.2	2.6 ±0.5
Hypoxanthine + ribose	0.9 ±0.1	0	0.5 ±0.2	83.2 ±2.2	7.0 ±1.1	4.7 ±0.2
Inosine	1.5 ±0.1	0	82.4 ±1.4	3.8 ±0.2	2.4 ±0.2	7.1 ±0.1

Values (means ± SE) represent radioactivity recovered in various high-pressure liquid chromatography fractions of perfusate. Uptake is calculated from ^{14}C-radiolabel measured in extracts of freeze-clamped hearts. Data are expressed as percent of [8-^{14}C]hypoxanthine or [8-^{14}C]inosine offered to hearts (n = 4–6). For statistical analysis, see text.

abolic fate of inosine and hypoxanthine was conversion to xanthine and uric acid. We determined [8-^{14}C]inosine or hypoxanthine conversion in the coronary effluent (Table 2). About 80% of both purines passed the heart unchanged and 10–15% were metabolized during a single passage through the coronary vasculature. About 0.8% of hypoxanthine was taken up by the heart, whether or not ribose was added. On the other hand, [8-^{14}C]inosine uptake was about 1.5%, which is twice the hypoxanthine uptake ($P < 0.001$). Incorporation rates were comparable for hypoxanthine and inosine. During reperfusion, the uptake figures for these purines were comparable to the control values. In the statistical analysis of the uric acid data neither the interaction nor the reperfusion versus normoxic data were statistically significant ($P = 0.76$; $P = 0.60$, respectively). The three treatments, however, were significantly different ($P < 0.0001$); by Scheffé's method of multiple comparison a significant difference ($P < 0.001$) existed between the group treated with inosine and the groups perfused with hypoxanthine in the presence or absence of ribose. Furthermore, perfusion with a mixture of ribose and hypoxanthine increased the uric acid release significantly ($P < 0.05$) compared with hypoxanthine infusion.

Table 3 shows the data on energy-rich phosphates determined in freeze-clamped hearts. During ischemia, total adenine nucleotides (TAN), ATP, and creatine phosphate (CrP) fell (20, 30, and 50%, respectively). Two-way analysis of variance showed a borderline significant interaction between normoxic and reperfused hearts with respect to TAN ($P = 0.13$) and ATP ($P = 0.06$), i.e., the decrease of TAN and ATP due to ischemia and reperfusion disappeared when 0.02 mM inosine was added to the reperfusion buffer. CrP during reperfusion showed an overshoot in all groups studied ($P < 0.001$, interaction 0.71).

DISCUSSION

Theoretically nucleotide synthesis rates from radiolabeled purines should be calculated on the basis of specific activity of the intracellular purine compounds. However, we were unable to measure intracellular hypoxanthine and inosine concentrations. To overcome this problem, we assumed that the hypoxanthine and inosine concentrations in the heart cell were reflected by the concentrations in the coronary effluent. As is shown in Fig. 4, 5 min after ischemia inosine and hypoxanthine concentrations returned to normal values (<0.1 μM). Therefore we concluded that within 5 min after ischemia, inosine and hypoxanthine concentrations in the heart cell had also returned to control values, which are low in heart (<1 μM, see, e.g., Ref. 20). To minimize isotopic dilution, 0.02 mM [8-^{14}C]hypoxanthine or -inosine were used.

Hypoxanthine and inosine incorporation rates were about 0.4 nmol·min^{-1}·g dry wt^{-1} into the ATP and 0.1 into the GTP pool (8 and 2% of the ^{14}C radioactivity accumulated in the heart, respectively; see Table 1). In earlier experiments we showed that about 75% of [U-^{14}C]inosine (also labeled in the ribose ring) recovered in nucleotides was incorporated into the combined ATP and GTP pool both in normoxic and in reperfused hearts (7). Therefore we estimated that the total hypoxanthine and inosine incorporation into adenine and guanine nucleotides amounts to about 0.7 nmol·min^{-1}·g dry wt^{-1} during aerobic perfusion. After ischemia a twofold increase in the incorporation rates of these purines into ATP and GTP is seen. The total amount of radioactivity taken up by the heart is not changed, however. Thus the percentage of hypoxanthine and inosine in heart, con-

TABLE 3. *Concentrations of high-energy phosphates in freeze-clamped rat hearts*

Addition	TAN	ATP	ADP	AMP	GTP	CrP
			Normoxia			
None	28.2 ±1.0	22.9 ±1.2	4.8 ±0.3	0.5 ±0.2	0.9 ±0.1	27.5 ±2.8
Hypoxanthine	27.4 ±1.7	22.1 ±1.2	4.9 ±0.6	0.3 ±0.1	1.0 ±0.3	33.0 ±2.6
Hypoxanthine + ribose	28.6 ±1.6	22.9 ±1.3	5.3 ±0.4	0.5 ±0.1	0.9 ±0.1	38.1 ±3.8
Inosine	26.3 ±2.1	21.1 ±1.6	4.6 ±0.6	0.7 ±0.2	0.9 ±0.1	22.3 ±1.5
			Ischemia			
None	23.0 ±2.1	15.8 ±2.8	5.8 ±1.1	1.4 ±0.6	0.7 ±0.2	13.4 ±1.9
			Reperfusion			
None	21.5 ±1.3	17.6 ±1.2	3.4 ±0.3	0.5 ±0.2	0.7 ±0.2	44.9 ±7.5
Hypoxanthine	23.7 ±1.0	19.6 ±1.0	3.8 ±0.1	0.3 ±0.1	0.8 ±0.1	44.3 ±3.7
Hypoxanthine + ribose	24.5 ±2.0	19.5 ±2.1	4.5 ±0.3	0.5 ±0.1	0.7 ±0.1	44.7 ±9.2
Inosine	26.7 ±1.2	22.3 ±0.9	4.0 ±0.2	0.4 ±0.1	0.9 ±0.1	37.5 ±1.7

Values are means ± SE (n = 3–6) in μmol/g dry wt. Hearts were perfused as described in legend to Fig. 3. Ischemia refers to hearts preperfused for 20 min, with an additional 20 min of ischemia. For statistical analysis, see text. TAN, total adenine nucleotides.

verted to ATP and GTP, is increased. The rest of the ^{14}C label is found in the fraction containing the nucleosides and oxypurines (see Ref. 8). We have not measured myocardial nucleosides and oxypurines, but we assume that more than 80% of the myocardial purine fraction consists of hypoxanthine (during hypoxanthine infusion) or inosine (during inosine infusion), as reflected by the perfusate concentration (see Table 2). If hypoxanthine and inosine were evenly distributed over the myocardial cells and extracellular space, intracellular hypoxanthine or inosine concentrations are estimated to be minimally 15 μM. These concentrations are in the same order of magnitude as found in the perfusate. Therefore ATP and GTP biosynthesis is not limited by purine transport but is regulated intracellularly. According to Zimmer et al. (24), the NADP-to-NADPH ratio decreases during and after ischemia, and the hexose-monophosphate shunt is accelerated. As a result of this process, ribose-5-phosphate levels are increased, which in turn increases the PRPP concentration. Because hypoxanthine phosphorylation is PRPP dependent, this could give a reasonable explanation for increased salvage rates after ischemia. It is possible to bypass the hexose-monophosphate shunt by supplying ribose as a precursor for PRPP (25). Indeed after ribose infusion, a significant increase in salvage rates is seen, which is further enhanced after ischemia.

Namm (14) found that during normoxic perfusion 0.01 mM inosine or hypoxanthine is incorporated into isolated rat hearts at a rate of about 3 nmol·min^{-1}·g dry wt^{-1}. Using myocardial homogenates of the same species, Maguire et al. (13) measured hypoxanthine phosphoribosyltransferase (HPRT) activities with a maximal initial velocity (V_{max}) of about 40 μmol·min^{-1}·g dry wt^{-1}. This is about 10,000 times higher than the salvage rates reported by Namm (14) or found in our experiments. Although an in vitro enzyme activity measurement can hardly be compared with in vivo activity, it seems that the hypoxanthine incorporation is not limited by the potentially available HPRT activity. Raivio et al. (15) have found a striking correlation between PRPP synthesis and cellular phosphate concentration in human fibroblasts. Myocardial PRPP synthesis could be regulated by phosphate concentration as well.

During hypoxanthine perfusion (with or without ribose) after ischemia, a slight (NS) increase of TAN is seen. During postischemic inosine infusion, however, TAN and ATP are about 5 μmol/g dry wt higher. ATP biosynthesis from [8-^{14}C]inosine during reperfusion is 1.1 nmol·min^{-1}·g dry wt^{-1} (Table 1), so TAN synthesis will be about 1.5. This is insufficient by far to account for the observed total restoration. There are two other known pathways that could account for ATP biosynthesis, namely adenosine phosphorylation and de novo synthesis. Adenosine phosphorylation accounts maximally for 50 nmol·min^{-1}·g dry wt^{-1} (14, 16). However, the adenosine concentration in the perfusate at the end of the ischemic period is about 1.2 μmol/l and is lowered within 5 min of reperfusion to 0.04 μmol/l. Therefore, under these experimental conditions, adenosine phosphorylation will be low because of lack of substrate. De novo synthesis in isolated rat hearts is 0.1 nmol·min^{-1}·g dry wt^{-1} (23). This synthesis rate is PRPP dependent and is accelerated after ischemia. Whether de novo synthesis is stimulated by inosine is unknown at this moment, but it seems unrealistic to assume that the restoration of TAN and ATP is due to de novo synthesis. Bates et al. (1) postulated the existence of a pool of nonacid-extractable ATP, which under certain conditions can be converted to acid-extractable ATP. It remains to be seen whether this explanation is applicable to our results.

During reperfusion an overshoot of creatine phosphate is seen (Table 3). In the model of Saks et al. (17) CrP serves as an "energy carrier" from mitochondria to sites of utilization. In this scheme it is possible for the mitochondria to start producing energy after mild ischemia, without direct utilization. For that reason, no CrP overshoot would be expected after severe ischemia, when the mitochondria are damaged (see Ref. 18).

To our knowledge, this is the first report describing purine incorporation into the myocardial GTP pool, although Swain et al. (20) recently reported restoration of GTP levels by de novo synthesis in dog heart. It is interesting to see that the purine incorporation rate into GTP is about 25% of that of ATP, although the GTP content in heart is only about 5% of the ATP content. This indicates a faster recovery of GTP after ischemia.

Our final conclusions are that hypoxanthine and inosine are incorporated in both the ATP and GTP pool in the heart. This process is stimulated after ischemia and by ribose perfusion and is thereby dependent on myocardial PRPP concentrations. Inosine especially seems to restore ATP levels, and this could (partly) explain its beneficial action.

The authors thank J. Tijssen from the Clinical Epidemiology Unit of the Thoraxcenter for performing the statistical analysis.

Received 22 March 1983; accepted in final form 30 August 1983.

REFERENCES

1. BATES, D. J., D. PERRETT, AND J. MOWBRAY. Systemic variations in the content of purine nucleotides in the steady-state perfused rat heart. Evidence for the existence of controlled storage and release of adenine-nucleotides. *Biochem. J.* 176: 485–493, 1978.
2. DE JONG, J. W. Biochemistry of acutely ischemic myocardium. In: *The Pathophysiology of Myocardial Perfusion*, edited by W. Schaper. Amsterdam, the Netherlands: Elsevier/North-Holland Biomedical, 1979, p. 719–750.
3. DE JONG, J. W., E. HARMSEN, P. P. DE TOMBE, AND E. KEIJZER. Nifedipine reduces adenine nucleotide breakdown in ischemic rat heart. *Eur. J. Pharmacol.* 81: 89–96, 1982.
4. GOLDHABER, S. Z., G. M. POHOST, R. A. KLONER, E. ANDREWS, J. B. NEWELL, AND J. S. INGWALL. Inosine: a protective agent in an organ culture of myocardial ischemia. *Circ. Res.* 51: 181–188, 1982.
5. GOLDTWAITH, D. A. Mechanisms of synthesis of purine nucleotides in heart muscle extracts. *J. Clin. Invest.* 36: 1572–1578, 1957.
6. HARMSEN, E., J. W. DE JONG, AND P. W. SERRUYS. Hypoxanthine production by ischemic heart demonstrated by high pressure liquid chromatography of blood purine nucleosides and oxypurines. *Clin. Chim. Acta* 115: 73–84, 1981.
7. HARMSEN, E., P. P. DE TOMBE, P. W. ACHTERBERG, AND J. W. DE JONG. Inosine incorporation into adenine- and guanine nucleotides in the isolated perfused rat heart is stimulated after ischemia.

Adv. Exp. Med. Biol. 48: 487–490, 1983.
8. HARMSEN, E., P. P. DE TOMBE, AND J. W. DE JONG. Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography. *J. Chromatogr.* 230: 131–136, 1982.
9. JENNINGS, R. B., K. A. REIMER, M. L. HILL, AND S. E. MAYER. Total ischemia in dog hearts, in vitro. *Circ. Res.* 49: 892–900, 1981.
10. JONES, C. E., AND L. R. MAYER. Nonmetabolically coupled coronary vasodilatation during inosine infusion in dogs. *Am. J. Physiol.* 238 (*Heart Circ. Physiol.* 7): H569–H574, 1980.
11. JUHASZ-NAGY, A., AND D. M. AVIODO. Inosine as a cardiotonic agent that reverses adrenergic beta blockade. *J. Pharmacol. Exp. Ther.* 202: 683–695, 1977.
12. LIU, M. S., AND H. FEINBERG. Incorporation of adenosine-8-^{14}C and inosine-8-^{14}C into rabbit heart adenine nucleotides. *Am. J. Physiol.* 220: 1242–1248, 1971.
13. MAGUIRE, M. H., M. C. LUKAS, AND J. F. RETTIE. Adenine nucleotide salvage synthesis in the rat heart; pathways of adenosine salvage. *Biochim. Biophys. Acta* 262: 108–115, 1972.
14. NAMM, D. H. Myocardial nucleotide synthesis from purine bases and nucleosides. *Circ. Res.* 33: 686–695, 1973.
15. RAIVIO, K. R., C. S. LAZAR, AND M. A. BECKER. Regulation of 5-phosphoribosyl-1-pyrophosphate synthesis in human fibroblasts by the concentration of inorganic phosphate. *Biochim. Biophys. Acta* 678: 58–64, 1981.
16. REIBEL, D. K., AND M. J. ROVETTO. Myocardial adenosine salvage rates and restoration of ATP content following ischemia. *Am. J. Physiol.* 237 (*Heart Circ. Physiol.* 6): H247–H252, 1979.
17. SAKS, V. A., G. B. CHERNOUSOVA, I. I. VORONKOV, V. N. SMIRNOVA, AND E. I. CHAZOV. Study of energy transport mechanism in myocardial cells. *Circ. Res.* 34/35, *Suppl.* III: 138–149, 1974.
18. SCHAPER, J., J. MULCH, B. WINKLER, AND W. SCHAPER. Ultrastructural, functional, and biochemical criteria for estimation of reversibility of ischemic injury: a study on the effects of global ischemia on the isolated dog heart. *J. Mol. Cell. Cardiol.* 11: 521–541, 1979.
19. SNEDECOR, G., AND W. G. COCHRAN. *Statistical Methods* (6th ed.). Ames, IA: Iowa State Univ. Press, 1967, p. 299–338.
20. SWAIN, J. L., J. J. HINES, R. L. SABINA, AND E. W. HOLMES. Accelerated repletion of ATP and GTP pools in postischemic canine myocardium using a precursor of purine de novo synthesis. *Circ. Res.* 51: 102–105, 1982.
21. THOMAS, J. X., AND C. E. JONES. Effect of inosine on contractile force and high-energy phosphates in ischemic hearts. *Proc. Soc. Exp. Biol. Med.* 161: 468–472, 1979.
22. WIEDMEIER, V. T., R. RUBIO, AND R. M. BERNE. Inosine incorporation into myocardial nucleotides. *J. Mol. Cell. Cardiol.* 4: 445–452, 1972.
23. WOOLLARD, K. V., R. O. KINGABY, M. J. LAB, A. W. G. COLE, AND T. N. PALMER. Inosine as a selective inotropic agent on ischemic myocardium? *Cardiovasc. Res.* 15: 659–667, 1981.
24. ZIMMER, H.-G., C. TRENDELENBURG, H. KAMMERMEIER, AND E. GERLACH. De novo synthesis of myocardial adenine nucleotides in the rat. *Circ. Res.* 32: 635–642, 1973.
25. ZIMMER, H.-G., H. IBEL, G. STEINKOPFF, AND G. KORB. Reduction of the isoproterenol-induced alterations in cardiac adenine nucleotides and morphology by ribose. *Science* 207: 319–321, 1980.

APPENDIX PAPER

IV

Myocardial S-adenosylhomocysteine hydrolase is important for adenosine production during normoxia

Biochimica et Biophysica Acta 840 (1985) 393–400
Elsevier

BBA 22071

Myocardial *S*-adenosylhomocysteine hydrolase is important for adenosine production during normoxia

Peter W. Achterberg, Peter P. de Tombe *, Eef Harmsen **
and Jan Willem de Jong

Cardiochemical Laboratory, Thorax Center, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

(Received December 10th, 1984)
(Revised manuscript received March 25th, 1985)

Key words: *S*-Adenosylhomocysteine hydrolase; Vasodilator; Adenosine production; Myocardial ischemia; HPLC; (Rat heart)

(1) The coronary vasodilator adenosine can be formed in the heart by breakdown of AMP or *S*-adenosylhomocysteine (*S*AdoHcy). The purpose of this study was to get insight into the relative importance of these routes of adenosine formation in both the normoxic and the ischemic heart. (2) A novel HPLC method was used to determine myocardial adenosine and *S*AdoHcy. Accumulation of *S*AdoHcy was induced in isolated rat hearts by perfusion with L-homocysteine thiolactone or L-homocysteine. The release of adenosine, inosine, hypoxanthine, xanthine and uric acid was determined. Additional in vitro experiments were performed to determine the kinetic parameters of *S*-adenosylhomocysteine hydrolase. (3) During normoxia the thiolactone caused a concentration-dependent increase in *S*AdoHcy. At 2000 μM of the thiolactone an *S*AdoHcy accumulation of 0.49 nmol/min per g wet weight was found during normoxia. L-Homocysteine (200 μM) caused an increase of 0.37 and 4.17 nmol *S*AdoHcy/min per g wet weight during normoxia and ischemia, respectively. (4) The adenosine concentration in ischemic hearts was significantly lower when homocysteine was infused (6.2 vs. 11.5 nmol/g; $P < 0.05$). Purine release was increased 4-fold during ischemia. (5) The K_m for hydrolysis of *S*AdoHcy was about 12 μM. At in vitro conditions favoring near-maximal *S*AdoHcy synthesis (72 μM adenosine, 1.8 mM homocysteine), the synthesis rate in homogenates was 10 nmol/min per g wet weight. (6) From the combined in vitro and perfusion studies, we conclude that *S*-adenosylhomocysteine hydrolase can contribute significantly to adenosine production in normoxic rat heart, but not during ischemia.

Introduction

Myocardial adenosine has been implied as metabolic regulator of coronary flow [1,2] and as attenuator of catecholaminergic effects during myocardial ischemia [3]. Chronotropic and dromotropic influences of adenosine on the heart have

* Present address: Department of Medicine and Medical Physiology, Foothills Hospital, University of Calgary, 1203 29th Street, N.W. Calgary, Alberta T2N2T9, Canada.
** Present address: Department of Biochemistry, University of Oxford, South Park Road, Oxford OX1 3QU, U.K.

Abbreviations: *S*AdoHcy, *S*-adenosylhomocysteine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine-HCl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

also been reported [4], as well as influences on adenylate cyclase [5], possibly via specific adenosine receptors [6,7]. Formation of adenosine can occur either via hydrolysis of AMP [8] or via hydrolysis of *S*-adenosylhomocysteine (*S*AdoHcy) by the enzyme *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1). This last reaction is reversible with equilibrium lying in the direction of *S*AdoHcy synthesis [9]. However, adenosine formed by the heart is quickly broken down into inosine and further into hypoxanthine, and in most species into xanthine and uric acid [10]. Adenosine can also be removed by phosphorylation to AMP [11]. Because the homocysteine concentration, the second reactant in the *S*-adenosylhomocysteine hydrolase reaction, is normally low and adenosine is removed quickly, any adenosylhomocysteine that is formed in the cellular methylation cycle [12] will be hydrolyzed to adenosine (Fig. 1). Schrader and co-workers [8,13] have investigated the properties of *S*-adenosylhomocysteine hydrolase from guinea pig heart. *S*-Adenosylhomocysteine hydrolase activity in rat heart was found to be several-fold lower than in guinea pig heart [13].

We have perfused isolated rat hearts with L-homocysteine or homocysteine thiolactone in order to trap intramyocardially produced adenosine, both during normoxia and during low flow ischemia, when adenosine formation is increased. The results of perfusion studies are combined with those of in vitro studies in which we have determined the kinetic parameters of *S*-adenosylhomocysteine hydrolase. We suggest an important role for this enzyme in the regulation of the adenosine concentration in the normoxic, but not in the ischemic heart. Preliminary results of this study have been published in abstract form [14].

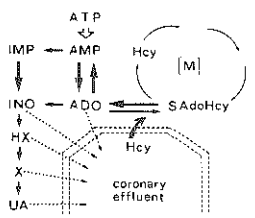


Fig. 1. Pathways of myocardial purine metabolism. M, methylation cycle; Hcy, homocysteine; *S*AdoHcy, *S*-adenosylhomocysteine; ADO, adenosine; INO, inosine; HX, hypoxanthine; X, xanthine; UA, uric acid.

Materials and Methods

Chemicals

All chemicals were analytical grade. Water (used for HPLC buffers and perfusion and assay media) was purified with the Milli-Ro4/Milli-Q system (Millipore Co., Bedford, MA). L-Homocysteine was prepared by alkaline hydrolysis of L-homocysteine thiolactone according to Duerre and Miller [15] for perfusion studies, or according to Ueland [16] for in vitro studies. In the latter KOH was used instead of NaOH, with neutralization to pH 7.4 instead of 7.0. L-Homocysteine thiolactone was obtained from Sigma (St. Louis, MO). EHNA was purchased from Burroughs Wellcome, Research Triangle Park, NC.

Perfusion of rat hearts

Hearts from male Wistar rats (275–350 gram), fed ad libitum, were excised and retrogradely perfused as described before [17]. Perfusion medium consisted of a modified Tyrode's solution, containing 125 mM NaCl, 4.7 mM KCl, 1.35 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCl_2$ and 10 mM glucose. It was gassed with 95% O_2/5% CO_2. Perfusion pressure was 72 mmHg. The temperature, measured in the cannula tip, was maintained at 37.0 ± 0.5°C. Ventricles were paced (4 V, 2 ms) at 300 beats/min. In the experiments where homocysteine was infused, coronary flow was kept between 9 and 10 ml/min by a rollerpump (LKB, Stockholm, Sweden, model 2132) for an equilibration period of 15 min. Homocysteine was kept in perfusion medium gassed with 95% N_2/5% CO_2 in order to prevent oxidation and it was infused into the perfusion system less than 5 s before it entered the heart. The final homocysteine concentration was 200 μM with 85% O_2/10% N_2/5% CO_2. Ischemia was induced by reducing coronary flow to 2.0–2.5 ml/min via the rollerpump. Perfusion with homocysteine was continued for 20 min. Controls did not receive homocysteine, but were otherwise treated identically (85% O_2, etc.).

When homocysteine thiolactone (100–2000 μM) was infused in a preliminary series of experiments,

equilibration time was also 15 min, but coronary flow was not regulated by a pump, and infusion of the thiolactone lasted 15 min.

Assay of purine compounds

Adenosine, inosine, hypoxanthine, xanthine and uric acid that were released from the hearts into the perfusates were determined as described previously [18]. Myocardial adenosine and *S*AdoHcy were determined in neutralized perchloric acid extracts of hearts that were freeze-clamped at the end of the experiments. The extracts were prepared as described for adenine nucleotide determination [19]. Neutralized extracts (200 μl) were injected onto a Partisil SAX column (Whatman, Maidstone, U.K.) and eluted with 19 mM KH_2PO_4 (pH 2.85; flow, 2 ml/min). Adenosine and *S*AdoHcy eluted between 1 and 2 min. They were collected in a 2-ml sample loop, injected again onto a μBondapak C_{18} column (Waters Associates, Milford, MA) and eluted with a KH_2PO_4 buffer (70 mM, 3% methanol added, pH 4.5) at a flow of 3 ml/min. The optimal time between injection onto the first column and switching of the sample loop to the second column was determined with the use of standards. Moreover, *S*AdoHcy standards have been treated as in the procedure used for extraction of rat hearts. Recoveries were greater than 95%.

*S*AdoHcy was also determined in samples of in vitro assays by the HPLC system for purine analysis in perfusates (see next paragraph).

In vitro studies on rat heart SAdoHcy

Direction of SAdoHcy hydrolysis. *S*-adenosylhomocysteine hydrolase activity in direction of hydrolysis was studied in preparations of rat heart, which were partially purified by ammonium sulfate precipitation [20]. Enzyme activity was measured spectrophotometrically via the enzyme-cascade method described by Schütz and collaborators [8]. K_m and V_{max} were determined in two separate experiments. The *S*AdoHcy concentration was varied (1–21 μM) and activity per mg protein was determined. Lineweaver-Burk plots were used to determine K_m and V_{max} values. Protein was determined using the Bio-Rad (Munich, F.R.G) protein assay [21], with bovine serum albumin as standard.

Direction of SAdoHcy synthesis. Rat hearts were homogenized in 10 vol. (ml/g wet wt.) of Hepes/KOH (10 mM, pH 7.4; 2 mM $MgCl_2$, 1 mM dithiothreitol). Filtration over nylon gauze was followed by centrifugation and removal of low molecular weight compounds by gel-filtration over a PD-10 column (Pharmacia, Uppsala, Sweden). Assay mixtures contained 1.8 mM homocysteine, 72 μM adenosine and 5 μM EHNA (final volume, 2.4 ml), the latter to prevent breakdown of adenosine by adenosine deaminase. Various amounts of homogenate were incubated at 37°C in the homogenization buffer. Samples (1 ml) were taken at different time intervals and were added to 1 ml ice-cold perchloric acid (8%). Protein was removed by centrifugation. After neutralization with KOH and removal of $KClO_4$ by centrifugation (0°C), samples were injected on the HPLC system [18] described for purine determination.

Statistics

Statistical significance was evaluated by Student's *t*-test for paired or unpaired observations and P values (two-tailed) were calculated. $P \geqslant 0.05$ was considered not significant. Results are expressed as means ± S.E. The number of experiments is represented by n.

Results

Perfusion of isolated rat hearts

Effects of homocysteine thiolactone infusion. Homocysteine thiolactone was infused at concentrations of 0, 100, 430 and 2000 μM. Fig. 2 shows the myocardial *S*AdoHcy and adenosine after 15 min of perfusion with the thiolactone. *S*AdoHcy increased with increasing thiolactone concentrations, while myocardial adenosine concentrations increased to control levels after an initial decrease at 100 μM of the thiolactone (Fig. 2). Table I gives the data on purine release from these hearts over the 15 min period of perfusion with thiolactone. Homocysteine thiolactone (100 μM) caused a decrease in purine release (Table I), but this was far greater than the observed intramyocardial *S*AdoHcy accumulation (Fig. 2). At 430 μM homocysteine thiolactone the purine release appeared to be equal to control level. Purine release at 2000 μM thiolactone could not be determined because

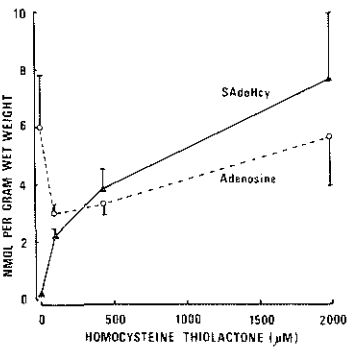


Fig. 2. Effect of homocysteine thiolactone on intramyocardial accumulation of adenosine and *S*AdoHcy. Isolated rat hearts were retrogradely perfused (pacing, 300 beats/min). After 15 min equilibration, hearts were perfused with homocysteine thiolactone for 15 min and freeze-clamped at the end of the experiments. Adenosine and *S*AdoHcy were determined in neutralized perchloric acid extracts of the hearts. ○, adenosine; ▲, *S*AdoHcy. Values are means ± S.E. of four or five hearts.

of the appearance of unidentified peaks in our chromatograms. We suggest that metabolic disturbances occur, when high concentrations of homocysteine thiolactone are infused (see Discussion).

Effects of homocysteine infusion. In Table II results are presented of rat heart perfusions with 200 μM homocysteine during normoxia and ischemia. Special care was given to prevent oxidative degradation of homocysteine before it entered the heart. Ischemia induced a 4-fold increase in purine release in both homocysteine-treated and non-treated groups. Total purine release during normoxia, as well as during ischemia was not influenced by homocysteine infusion. In normoxic hearts *S*AdoHcy levels were low (0.2 nmol/g wet wt.) and they increased to 7.6 nmol/g wet wt., when homocysteine was present. During ischemia homocysteine increased intramyocardial *S*AdoHcy from 0.6 to 84 nmol/g wet wt. during the 20 min perfusion period. This reflects the greater availability of adenosine during ischemia. Sequestration of myocardial adenosine by homocysteine was also suggested by the fact that the ischemic adenosine concentration (11.5 nmol/g) was significantly lower in the presence of homocysteine (6.2 nmol/g; $P < 0.05$).

In vitro studies

A dialyzed supernatant after 35% ammonium sulfate precipitation had an activity in the direction of *S*AdoHcy hydrolysis of 0.19 nmol/min per mg protein ([*S*AdoHcy] = 20 μM). The dialyzed, resuspended pellet of a 60% ammonium sulfate precipitate gave an activity of 0.51 nmol/min per mg protein. The K_m of *S*AdoHcy in direction of hydrolysis was found to be 12–13 μM with a V_{max} value of 0.7–0.8 nmol/min per mg protein. The latter is equivalent to 18–20 nmol/min per g wet wt.

Under conditions of maximal *S*AdoHcy synthesis (i.e., 1.8 mM homocysteine, 72 μM adenosine, 5 μM EHNA and 37°C), the reaction was linear for only 15 min. Inactivation of the enzyme might be the cause [22]. The reaction was linear with the amount of enzyme and two separate determinations gave an *S*AdoHcy synthesis rate of 9.6–10.4 nmol/min per g wet wt.

TABLE I

EFFECT OF HOMOCYSTEINE THIOLACTONE ON NORMOXIC MYOCARDIAL PURINE RELEASE

Hearts were perfused as described in the legend to Fig. 2. Perfusates were collected on ice over 15 min, and purines were determined with HPLC. *n*, number of experiments; HcyT, homocysteine thiolactone; Ado, adenosine; Ino, inosine; Hx, hypoxanthine; X, xanthine; UA, uric acid. Values are expressed as nmol/15 min per g wet weight ± S.E.

HcyT (μM)	*n*	Ado	Ino	Hx	X	UA	Total purines
0	5	1.8 ± 0.8	5.8 ± 0.8	2.8 ± 0.8	3.6 ± 1.0	28 ± 6	42 ± 4
100	5	0.9 ± 0.2	3.7 ± 0.9	2.3 ± 0.5	3.6 ± 0.5	10.9 ± 1.0 [a]	21 ± 3 [a]
430	4	0.3 ± 0.3	9 ± 3	1.3 ± 0.9	4.7 ± 1.4	37 ± 5	52 ± 7

[a] $P < 0.05$ vs. 0 μM homocysteine thiolactone.

Discussion

Homocysteine thiolactone versus homocysteine infusion

Because of the fact that L-homocysteine will be oxidized when dissolved in oxygenated perfusion medium, we started our experiments with homocysteine thiolactone. The thiolactone is converted intracellularly to homocysteine. At higher concentrations, however, the thiolactone appeared to be toxic to the hearts. The occurence of acidosis, caused by intracellular hydrolysis of high amounts of the thiolactone, might be a factor responsible for this [23]. The observed decrease of purine release upon infusion of 100 μM thiolactone (Table I) cannot be explained by the increase in intramyocardial *S*AdoHcy. L-Homocysteine (200 μM), which we used for further experiments, gave a similar, although nonsignificant reduction of purine release (Table II) during normoxia. A possible explanation for the effects observed at lower concentrations of both compounds is reduction of glutathione by homocysteine [24], which might lead to alterations in myocardial oxidation/reduction state and metabolism [25].

Significant adenosine production from SAdoHcy during normoxia

Observations from our perfusion studies with homocysteine can be compared to the kinetic parameters of *S*AdoHcy hydrolase obtained from in vitro studies that are reported here and by other authors. We assume simple Michaelis-Menten kinetics to be applicable.

The *S*AdoHcy concentrations in normoxic rat heart was found to be 0.2–0.6 nmol per g wet wt. This gives an estimated *S*AdoHcy concentration of 0.4–1.5 μM. The K_m for *S*AdoHcy hydrolysis was 12–13 μM with a V_{max} of 18–20 nmol/min per g wet wt. The synthesis of adenosine from *S*AdoHcy in the absence of homocysteine can then be calculated to vary between 0.5 and 2.2 nmol/min per g wet wt. From measurements of rat heart methylation cycle [12] a value of 0.7 nmol/min per g wet wt. was calculated, which is in good agreement with our estimate. Adenosine production during normoxia in the presence of homocysteine (200 μM) can be estimated from *S*AdoHcy accumulation to be at least 0.38 nmol/min per g wet wt., but more than 10-fold higher (4.2 nmol/min per g) during ischemia.

Normoxic purine release amounted to 7 ± 4 nmol/min per g wet wt. and was increased 3-fold during ischemia (20 nmol/min per g; Table II). Considering the kinetic parameters of the enzyme in the direction of synthesis [14] and of adenosine deaminase, one would not expect a more than 10-fold rise in *S*AdoHcy accumulation, due to homocysteine infusion during ischemia with a simultaneous 4-fold rise in total purine release. The K_m (adenosine) of *S*-adenosylhomocysteinase is lower than 1 μM [15], while the K_m (adenosine) for adenosine deaminase is about 50 μM. This observed difference can be explained by assuming extremely low normoxic adenosine concentrations in the cellular compartment (cytosol) that contains adenosine deaminase and *S*-adenosylhomocysteine hydrolase, for instance caused by the reported binding of adenosine to *S*-adenosylhomocysteine hydrolase [26]. In that case it has to be assumed that normoxic formation of purines takes place for a major part via AMP → IMP → inosine and therefore bypasses adenosine formation. Other studies in our laboratory have recently supported this hypothesis: we have reported experiments [27] in which we infused a strong inhibitor of adenosine deaminase into isolated rat hearts at a concentration which inhibited the deamination of infused adenosine for more than 90%. The inhibitor (EHNA, 50 μM) increased the relative release of adenosine during normoxia from 7 to 18% ($P < 0.05$) without changing total purine release. During ischemia EHNA increased adenosine release 3–4-fold, i.e., to 60% of total purine release. These results suggest that purine formation by way of AMP → IMP → inosine could be of significance during ischemia, but could be especially important during normoxia [27]. The same has also been suggested for guinea pig heart [28]. Furthermore, the AMP concentration in normoxic myocardial cytosol is extremely low (1 μM) [29,30] and thereby limits adenosine formation from AMP simply by lack of substrate. From evidence that virtually no rephosphorylation of adenosine by adenosine kinase could be detected in normoxic rat heart [31], it can be concluded that normoxic free adenosine concentration is extremely low. Moreover, all evidence which suggests that AMP →

TABLE II

PURINE RELEASE AND ACCUMULATION DURING NORMOXIC AND ISCHEMIC PERFUSION OF ISOLATED RAT HEARTS WITH HOMOCYSTEINE

Hearts were paced at 300 beats/min. Coronary flow was regulated with a roller pump at 9–10 ml/min. 15 min of preperfusion was followed by either reduction of flow (2.0–2.5 ml/min, ischemia) or continuation of flow (normoxia). Hearts were either perfused with 200 μM L-homocysteine or without addition. Perfusates were collected over the experimental time-period (20 min) after which the hearts were freeze-clamped. Purines were determined in perfusates, and adenosine and *S*AdoHcy in extracts of freeze-clamped hearts. Results are expressed as means ± S.E. ($n = 4$, except for ischemic controls ($n = 3$)).

Conditions of perfusion	Purines released from the hearts (nmol/20 min per g wet wt.)						Intramyocardial accumulation (nmol per gram wet wt.)	
	adenosine	inosine	hypoxanthine	xanthine	uric acid	total purines	adenosine	*S*AdoHcy
Normoxia								
Controls	2 ± 2	14 ± 2	13 ± 4	10 ± 3	99 ± 14	138 ± 21	2.1 ± 0.6	0.2 ± 0.1
Homocysteine	1 ± 1	8 ± 1	5 ± 3	2 ± 1	76 ± 18	91 ± 19	4.0 ± 1.1	7.6 ± 0.5 [a]
Ischemia								
Controls	33 ± 4 [b]	131 ± 29 [b]	45 ± 7 [b]	36 ± 6 [b]	165 ± 34	410 ± 78 [b]	11.5 ± 0.7 [b]	0.6 ± 0.1
Homocysteine	18 ± 8	84 ± 21 [b]	51 ± 10 [b]	43 ± 7 [b]	194 ± 31 [b]	391 ± 71 [b]	6.2 ± 0.3 [a]	84 ± 5 [a,b]

[a] $P < 0.05$ vs. controls.
[b] $P < 0.05$ vs. normoxia.

adenosine is a major catabolic pathway in the heart, is solely referring to conditions where the AMP concentration has been elevated (e.g., ischemia, hypoxia, increased work load, acetate infusion). The arguments mentioned here tend to decrease the importance of AMP as a source of adenosine formation during normoxia and thereby emphasize the relative importance of *S*AdoHcy as a source of normoxic myocardial adenosine production.

Minor adenosine production from SAdoHcy during ischemia

K_m values for homocysteine (direction of *S*AdoHcy synthesis) have been reported to be 80–160 μM [32,33] and 260 μM [13]. From our perfusion experiments, we calculated the *S*AdoHcy synthesis rate in ischemic hearts to be 4.2 nmol/min per g wet wt. In our in vitro assays (at saturating adenosine and homocysteine concentrations) we determined a V_{max} value of 10 nmol/min per g wet wt. These values are in good agreement, if we assume ischemic myocardial adenosine concentrations to be well above K_m, and the homocysteine concentration (200 μM) in the range of K_m.

The fact that ischemic purine release was not significantly altered in the presence of homocysteine demonstrates a minor contribution of *S*AdoHcy hydrolysis to purine formation in the isolated ischemic rat heart. No significant decrease of adenosine release could be detected in our experiments with ischemic hearts that were perfused with homocysteine. Schrader et al. [13] found a significant decrease of adenosine release from hypoxic (30% O_2) guinea pig hearts perfused with homocysteine thiolactone. This difference can be explained by the fact that *S*-adenosylhomocysteine hydrolase activity in guinea pig heart is 6-fold higher than that in rat heart [13].

Concluding remarks

The fact that homocysteine infusion during ischemia gave a significant decrease of intramyocardial adenosine concentration without altering total purine release also supports the hypothesis that purine formation could occur in part via IMP during ischemia. In conclusion: if we assume (Refs. 27, 28 and this study) that during normoxia a considerable part of myocardial purines is formed via AMP → IMP → inosine (see Fig. 1) and that a significant amount of intracellular adenosine is bound [26,34] to the enzyme (*S*-adenosylhomocysteine hydrolase), we can conclude that rat heart *S*-adenosylhomocysteine hydrolase could play an important role in the regulation of normoxic intracellular adenosine formation and concentration.

Acknowledgements

The authors like to thank Mrs. M.J. Kanters-Stam for typing the manuscript and Mr. T.C. Verwoerd for his technical assistance. P.W.A. was supported by the Dutch Foundation for Medical Research (FUNGO).

References

1 Berne, R.M. (1963) Am. J. Physiol. 204, 317–322
2 Olsson, R.A., Snow, J.K. and Gentry, M.K. (1978) Circ. Res. 42, 358–362
3 Schrader, J., Baumann, G. and Gerlach, E. (1977) Pflügers Arch. 372, 29–35
4 Belardinelli, L., Belloni, F.L., Rubio, R. and Berne, R.M. (1980) Circ. Res. 47, 684–691
5 Londos, C., Cooper, D.M.F., Schlegel, W. and Rodbell, M. (1978) Proc. Natl. Acad. Sci. USA 75, 5362–5366
6 Olsson, R.A., Davis, C.J., Khouri, E.M. and Patterson, R. (1976) Circ. Res. 39, 93–98
7 Schrader, J., Nees, S. and Gerlach, E. (1977) Pflügers Arch. 369, 251–257
8 Schütz, W., Schrader, J. and Gerlach, E. (1981) Am. J. Physiol. 240, H963–H970
9 De La Haba, G. and Cantoni, G.L. (1959) J. Biol. Chem. 234, 603–608
10 Schoutsen, B., De Jong, J.W., Harmsen, E., De Tombe, P.P. and Achterberg, P.W. (1983) Biochim. Biophys. Acta 762, 519–524
11 Reibel, D.K. and Rovetto, M.J. (1979) Am. J. Physiol. 237, H247–H252
12 Watkins, C.A. and Morgan, H.E. (1979) J. Biol. Chem. 254, 693–701
13 Schrader, J., Schütz, W. and Bardenheuer, H. (1981) Biochem. J. 196, 65–70
14 Achterberg, P.W., De Tombe, P.P., Harmsen, E. and De Jong, J.W. (1983) in Regulatory Function of Adenosine (Berne, R.M., Rall, T.W. and Rubio, R., eds.), (Abstr.) p. 499, Martinus Nijhoff, Dordrecht
15 Duerre, J.A. and Miller, C.H. (1966) Anal. Biochem. 17, 310–315
16 Ueland, P.M. (1982) Int. J. Biochem. 14, 207–213
17 Harmsen, E., De Tombe, P.P., De Jong, J.W. and Achterberg, P.W. (1984) Am. J. Physiol. 246, H37–H43
18 Harmsen, E., De Jong, J.W. and Serruys, P.W. (1981) Clin. Chim. Acta 115, 73–84

19 Harmsen, E., De Tombe, P.P. and De Jong, J.W. (1982) J. Chromatogr. 230, 131–136
20 Kajander, E.O. and Raina, A.M. (1981) Biochem. J. 193, 503–512
21 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
22 Kajander, E.O. (1982) Biochem. J. 205, 585–592
23 Dudman, N.P.B. and Wilcken, D.E.L. (1982) Biochem. Med. 27, 244–253
24 Racker, E. (1955) J. Biol. Chem. 217, 867–874
25 Zimmer, H.-G., Bünger, R., Koschine, H. and Steinkopff, G. (1981) J. Mol. Cell. Cardiol. 13, 531–535
26 Hershfield, M.S. and Kredich, N.M. (1978) Science 202, 757–760
27 Achterberg, P.W., Harmsen, E. and De Jong, J.W. (1985) Cardiovasc. Res., in the press
28 Schrader, J. (1983) in Regulatory Function of Adenosine (Berne, R.M., Rall, T.W. and Rubio, R., eds.), pp. 133–156, Martinus Nijhoff, Dordrecht
29 Bünger, R. and Soboll, S. (1983) in Regulatory Function of Adenosine (Berne, R.M., Rall, T.W. and Rubio, R., eds.), (Abstr.) p. 504, Martinus Nijhoff, Dordrecht
30 Soboll, S. and Bünger, R. (1981) Hoppe Seyler's Z. Physiol. Chem. 362, 125–132
31 Newby, A.C., Holmquist, C.A., Illingworth, J. and Pearson, J.D. (1983) Biochem. J. 214, 317–323
32 Fujioka, M. and Takata, Y. (1981) J. Biol. Chem. 256, 1631–1635
33 Briske-Anderson, M. and Duerre, J.A. (1982) Can. J. Biochem. 60, 118–123
34 Olsson, R.A., Vomacka, R.B. and Nixon, D.G. (1979) in Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides (Baer, H.P. and Drummond, G.I., eds.), pp. 297–304, Raven Press, New York

APPENDIX PAPER

V

Adenosine deaminase inhibition and myocardial purine release during normoxia and ischemia

Cardiovascular Research, 1985, **19**, 593–598

Adenosine deaminase inhibition and myocardial purine release during normoxia and ischaemia

PETER W ACHTERBERG,* EEF HARMSEN, AND JAN WILLEM DE JONG

From the Cardiochemical Laboratory, Thoraxcenter, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

SUMMARY Quantitative determination of myocardial adenosine formation and breakdown is necessary to gain insight into the mechanism and regulation of its physiological actions. Deamination of adenosine was studied in isolated perfused rat hearts by infusion of adenosine (1 to 20 μmol·litre^{-1}). All catabolites in the perfusates (inosine, hypoxanthine, xanthine and uric acid) were measured, as well as unchanged adenosine. Apparent uptake of adenosine was determined; it increased linearly with the concentration of adenosine infused. Adenosine was predominantly deaminated, even at low (1 μmol·litre^{-1}) concentration. The inhibitory capacity of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was determined, while 5 μmol·litre^{-1} adenosine was infused. EHNA inhibited the apparent adenosine deaminase activity for 62 and 92% at 5 and 50 μmol·litre^{-1}, respectively. When 50 μmol·litre^{-1} EHNA was infused into normoxic hearts, release of adenosine was significantly elevated, as was coronary flow. Induction of ischaemia increased total purine release four- to fivefold. Infusion of EHNA into ischaemic hearts did not alter total purine release, but adenosine release increased from 15 to 60% of total purines. However, when EHNA was present, a large part of total purine release still existed of inosine, hypoxanthine, xanthiner and uric acid. This was 83% during normoxia and 40% during ischaemia. These results suggest significant contribution of IMP and GMP breakdown to purine release from isolated perfused rat hearts.

Several aspects of adenosine metabolism are important to myocardial (patho)physiology. Regulation of coronary flow by adenosine[1 2] is probably the most important aspect. Furthermore, adenosine has been implied as attenuator of catecholaminergic effects in ischaemic heart.[5] Chronotropic and dromotropic influences of adenosine in heart have also been reported.[6] Salvage of adenosine[3 4] could be significant to preserve the myocardial ATP pool. Myocardial purine metabolism, however, is very complex. For example, several pathways can contribute to formation and breakdown of adenosine in the heart.[7] Adenosine can be formed via dephosphorylation of AMP by action of 5′-nucleotidase (EC 3.1.3.5) or alkaline and acid phosphatase (EC 3.1.3.1), respectively or from S-adenosylhomocysteine (SAH) by means of SAH-hydrolase (EC 3.3.1.1.). Dephosphorylation is probably the most important, especially during ischaemia, when myocardial AMP levels rise strongly.[8] Removal of adenosine can occur by rephosphorylation to AMP (by adenosine kinase, EC 2.7.1.20) or by deamination to inosine (by adenosine deaminase, EC 3.5.4.4.). Inosine can be broken down further to hypoxanthine, xanthine and uric acid (see fig 1).

Quantitative determination of adenosine formation and breakdown in the heart is important to gain insight into the mechanism and regulation of its physiological actions. It has been tacitly assumed that the release of adenosine, inosine and hypoxanthine from the heart is a good measure of myocardial adenosine production.[9–11] For several reasons this assumption could be wrong. It neglects adenosine kinase activity and it does not account for release of xanthine and uric acid, which can be relatively large, depending on the myocardial xanthine oxidase/dehydrogenase (EC 1.2.3.2/1.2.1.37) activity of the species used.[12] Furthermore, breakdown of adenine nucleotides in the heart might not only proceed by way of AMP→adenosine→inosine, but also by way of AMP→IMP→inosine or GMP→→→xanthine. In these

*To whom correspondence and reprint requests should be sent.

Key words: adenosine; adenosine deaminase; purine metabolism.

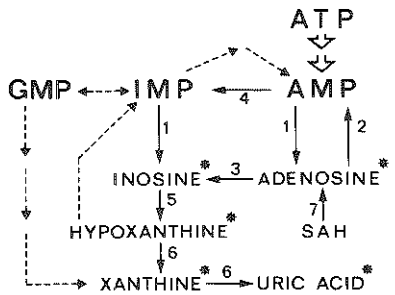


FIG 1 Pathways of purine metabolism
*Enzymes involved: 1 = 5'-nucleotidase; 2 = adenosine kinase; 3 = adenosine deaminase; 4 = AMP-deaminase; 5 = nucleoside phosphorylase; 6 = xanthine oxidase/dehydrogenase; 7 = SAH-hydrolase. SAH = S-adenosylhomocysteine. Dotted lines: quantitatively minor pathways or pathways not yet determined in the heart. *Purines which can be released from the heart.*

last two cases adenosine formation is bypassed and total purine release would not be a good measure of adenosine formation (fig 1).

One of the topics in myocardial adenosine metabolism over which some controversy exists, regards the rates at which infused adenosine is deaminated or incorporated into the heart. Jacob and Berne[13] have stressed the importance of phosphorylation, while De Jong[14] concluded that deamination was the most important fate of infused adenosine.

We report rates of deamination and uptake of adenosine infused into isolated rat hearts and the inhibitory capacity of the adenosine deaminase inhibitor EHNA. Finally, endogenous formation and breakdown of adenosine in normoxic and ischaemic rat heart was studied by infusion of EHNA. Our results suggest that not all purines released are produced via dephosphorylation of AMP to adenosine.

Materials and methods

CHEMICALS

All chemicals were analytical grade. Water (used for HPLC buffers and perfusion media) was purified with the Milli-Ro4/Milli-Q system (Millipore Co, Bedford, MA). EHNA (erythro-9-)2-hydroxy-3-nonyl) adenine.HCl) was obtained from Burroughs Wellcome, Research Triangle Park, NC.

PERFUSION OF HEARTS

Male Wistar rats (275 to 350 g), fed *ad libitum*, were used. After anaesthesia of the rats with pentobarbital (i.p.), hearts were quickly excised and immediately arrested in ice-cold saline or perfusion buffer, a modified Tyrode solution, containing 10 mmol·litre^{-1} glucose.[15] The Langendorff preparation was used with 100 cmH_2O perfusion pressure. Hearts were paced at 300 beats·min^{-1} (4 V stimulation for 2 ms). Perfusion temperature was kept at 37.0 ± 0.5°C. The medium was not recirculated and was gassed with 95% O_2, 5% CO_2. Ischaemia was induced by reducing perfusion pressure to 20 to 25 cmH_2O. At the end of the perfusion hearts were either taken from the apparatus and blotted dry for wet weight determination, or freeze clamped. In the case of freeze clamping, dry weight was determined by freeze drying. For comparison: under our conditions 1 g dry weight equals about 7 g wet wt.

CORONARY FLOW

Mean coronary flow was determined by collection of perfusate in a graduated cylinder over five-minute periods.

MYOCARDIAL FUNCTION

Apex displacement was used as an indicator of myocardial performance and measured as described previously.[16] It has been shown to correlate well with changes in myocardial pressure development during ischaemia and anoxia.[16] The displacement observed 5 min after the start of the perfusion was taken as 100%.

ASSAY OF PURINES

Adenosine, inosine, hypoxanthine, xanthine and uric acid were determined by a slightly modified version of the method described by Harmsen.[17] Briefly: 200 or 250 μl of perfusate were injected into a μBondapak C_{18} column (30 × 0.4 cm, Waters Assoc, Milford, MA) and eluted isocratically with 70 mm·litre^{-1} KH_2PO_4, pH 4.5, to which methanol was added. Flow rate was either 1 ml·min^{-1} (10% methanol added to buffer) or 3 ml·min^{-1} 3% methanol added). Peaks detected at 254 nm were compared with those of standards. Some ischaemic perfusates were incubated with uricase (EC 1.7.3.3) and deproteinised with perchloric acid. After neutralisation, the disappearance of the uric acid peak was established by HPLC.

DETERMINATION OF HIGH-ENERGY PHOSPHATES

ATP, ADP, and Creatine Phosphate were determined in neutralised, perchloric acid extracts of freeze-clamped hearts by the method of Harmsen.[18]

STATISTICAL ANALYSIS

Statistical significance was evaluated by Student's *t* test for paired or unpaired observations and p values (two-tailed) were calculated. $p>0.05$ was considered to be not significant. Results have been expressed as

means ± SEM. The number of experiments is represented by *n*.

Results

METABOLIC FATE OF INFUSED ADENOSINE

Infusion of increasing concentrations of adenosine (1 to 20 μmol·litre^{-1}) caused the release of increasing amounts of the catabolites inosine, hypoxanthine, xanthine and uric acid (fig 2). Inosine formation increased strongly with rising adenosine concentrations, while output of hypoxanthine, xanthine and uric acid rose more slowly. However, at a low adenosine concentration, the major pathway is still catabolism and the majority of catabolites(>60%) consists of xanthine and uric acid.

The sum of released catabolites reflects adenosine deaminase activity. This is shown in table 1, together with the (computed) apparent adenosine uptake. At all concentrations of adenosine studied deamination was found to be greater than uptake. Apparent uptake increased linearly with the amount of adenosine infused.

EFFECTS OF EHNA ON CATABOLISM OF INFUSED ADENOSINE

Table 2 shows the inhibition of apparent adenosine deaminase (ADA) activities by EHNA. Apparent

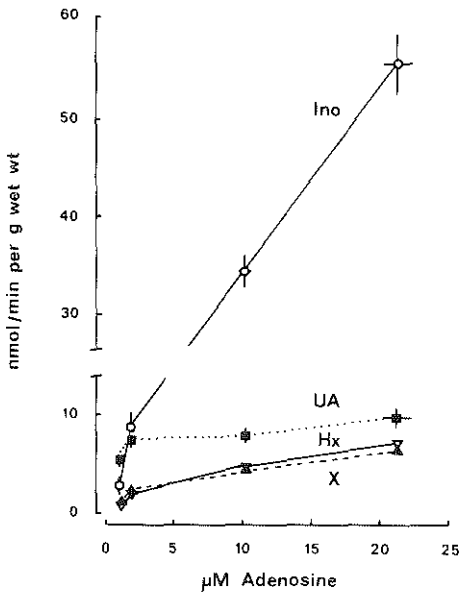


FIG 2 Formation of purines from adenosine infused into isolated rat hearts.
Isolated rat hearts were perfused with adenosine (two different concentrations per heart). Adenosine perfusions were preceded and followed by perfusion with medium alone. Each period of perfusion lasted ten minutes. Purines were determined in perfusates, collected during the second 5 min of each period. Values were corrected for average amounts of purines, released in the absence of adenosine. Points are means of 3 experiments ± SEM. If no bars are given, SEM's fall within the size of symbols. Ino = inosine (○); Hx = hypoxanthine (▽)) X = xanthine (▲); UA = uric acid (■).

TABLE 1 *Deamination and apparent uptake of adenosine infused into isolated rat hearts*

Adenosine input	*Unchanged adenosine*	*Catabolites* released*	*Apparent uptake*	*Coronary flow*
μmol·litre^{-1}	*(nmol·min^{-1}) per g wet wt*	*nmol·min^{-1} per g wet wt (% of input)*		*(ml·min^{-1}) per g wet wt*
1	16±3	3.3±0.4 (21%)	10±2 (63%)	2.7±0.9 (17%)	14±2)
2	32±8	9±2 (28%)	19±3 (59%)	3.5±0.5 (11%)	16±2
10	179±5	110±3 (62%)	52±2 (29%)	17±4 (10%)	17.4±0.3
20	400±10	286±48 (72%)	79±3 (20%)	31±12 (8%)	19±2

Isolated rat hearts were perfused as described in the legend to fig 2. Apparent uptake was computed from the difference in adenosine input and output of total purines (ie, unchanged adenosine plus catabolites). *Catabolites = sum of inosine, hypoxanthine, xanthine and uric acid; this reflects adenosine deamination. Means ± SEM of 3 experiments.

TABLE 2 *Apparent adenosine deaminase activities and inhibition by EHNA*

Expt	*EHNA* (μmol·litre^{-1})	*Adenosine infused*	*Apparent ADA activity*	*ADA inhibition* (%)
		(nmol·min^{-1} per g wet weight)		
I	0	107±7	42±3	0
	5	112.1±1.3	16.2±1.5*	62
II	0	105±7	49±3	0
	50	109±6	4.1±0.2*	92

After equilibration, adenosine (5 μmol·litre^{-1}) was infused into isolated rat hearts. After another equilibration period, adenosine was infused together with EHNA (Expt I: 5 μmol·litre^{-1} and Expt II: 50 μmol·litre^{-1}). Equilibration and infusion of adenosine and EHNA took place for 10 min periods. Purines were determined in perfusates, collected during the second 5 min of each period. Adenosine input was determined from the infused adenosine concentration. Apparent adenosine deaminase (ADA) activities were computed by summation of inosine, hypoxanthine, xanthine and uric acid release. Values were corrected for the average purine release before and after adenosine infusion. $\bar{x}$ ± SEM (n = 4): *$p<0.001$ *vs* 0 μmol·litre^{-1} EHNA.

ADA activities were computed by summation of release of inosine, hypoxanthine, xanthine and uric acid, corrected for control release of purines. EHNA (50 μmol·litre^{-1}) inhibited the deamination of 5 μmol·litre^{-1} adenosine by more than 90%.

EFFECTS OF EHNA DURING NORMOXIA AND ISCHAEMIA

Fig 3 shows the results of measurements of coronary flow and myocardial function (apex displacement) in hearts perfused with or without 50 μmol·litre^{-1} EHNA. When EHNA was infused during normoxia, apex displacement was not significantly changed. Normoxic coronary flow increased more than 50% ($p<0.05$) in the presence of EHNA. Reduction of perfusion pressure from 100 to 20 cmH_2O decreased coronary flow and reduced apex displacement in the absence as well as in the presence of EHNA.

Purine release from these hearts is shown in fig 4. During normoxia, EHNA induced a small but significant ($p<0.02$) rise in adenosine release, although the relative release of inosine, hypoxanthine, xanthine and uric acid remained high (80%). Total normoxic purine release was not altered. When hearts were made ischaemic, adenosine release was increased three- to fourfold by addition of EHNA ($p<0.02$). Release of inosine, hypoxanthine and xanthine was lower in the presence of EHNA ($p<0.02$). Uric acid release was also lower, but the difference was not statistically significant. Total ischaemic purine release was not altered by EHNA. These results indicate inhibition of myocardial adenosine deaminase activity during normoxia and ischaemia. Still a considerable amount of inosine, hypoxanthine, xanthine, but especially uric acid was released.

MYOCARDIAL HIGH-ENERGY PHOSPHATES

After 15 min of mild ischaemia (experiments of fig 4) the following levels of high-energy phosphates were found in the absence of EHNA: ATP, 20.4 ± 1.5; ADP, 5.3 ± 0.9; Creatine Phosphate, 24.9 ± 1.3 (μmol per g dry wt.; $\bar{x}$ ± SEM; n = 4). Addition of 50 μmol·litre^{-1} EHNA during ischaemia caused no significant differences: ATP, 23.8 ± 0.9; ADP 6.2 ± 0.4, and Creatine Phosphate, 25.7 ± 1.3 (μmol per g dry wt.).

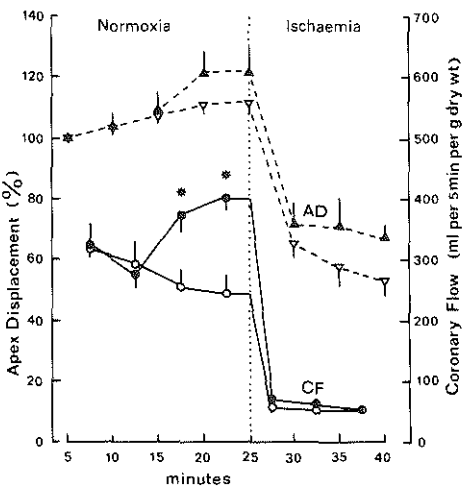


FIG 3 Effect of EHNA on coronary flow and apex displacement in normoxic and ischaemic hearts
After 15 min of equilibration, isolated rat hearts were perfused in the absence (○ and ▽) or presence (● and ▲) of 50 μmol·litre^{-1} EHNA. At t = 25 min hearts were made ischaemic by reduction of perfusion pressure. AD = apex displacement (▲ and ▽). CF = coronary flow (○ and ●). AD at t = 5 min was taken as 100%. Data are means of 4 experiments ± SEM. If no bars are given, SEM's fall within size of the symbols.
**$p<0.05$ vs non-treated.*

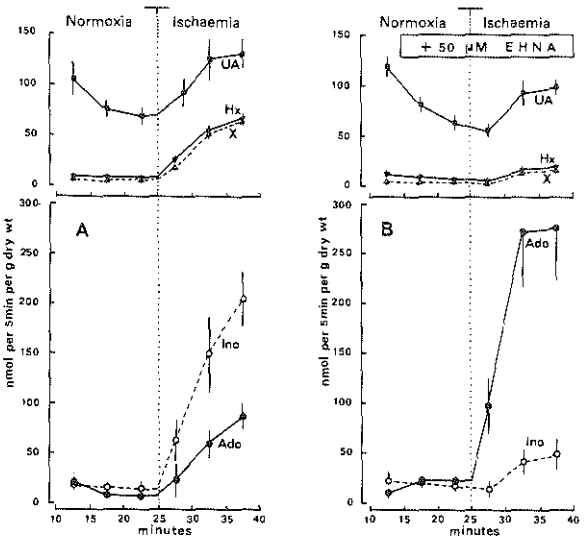


FIG 4 Effect of EHNA on release of purine nucleosides and oxypurines from normoxic and ischaemic rat hearts.
Isolated rat hearts were perfused in the absence (A) or presence (B) of 50 μmol·litre^{-1} EHNA as indicated in the legend to fig 3. Purines were determined in perfusates collected over 5 min periods. Purine nucleosides: *Ado = adenosine (●); Ino = inosine (○).* Oxypurines: *Hx = hypoxanthine (▼); X = xanthine (△); UA = uric acid (■). Values are means ± SEM (n = 4). Significant differences (and p values) between the groups of figs 4a and 4b are given in the results section.*

Discussion

In quantitative studies on myocardial purine metabolism, one has to make certain that all compounds contributing to purine efflux have been measured. In many studies, however, xanthine and/or uric acid have not been determined. Still, myocardial xanthine oxidase/dehydrogenase activity has been reported in man, cow, sheep, dog, cat and rat. For example, in the isolated perfused rat heart xanthine and uric acid make up 70% of purines released during normoxia.[12] Moreover, our experiments show that infusion of low amounts of adenosine (1 μmol·litre^{-1}) gives rise to 60 to 70% of catabolites recovered as xanthine and uric acid. Breakdown of exogenous adenosine up to uric acid in rat heart has been reported with 5 μmol·litre^{-1}.[19] Our results are in good agreement with that study. At all concentrations of adenosine studied, deamination was found to be greater than uptake, which increased linearly with the concentration of infused adenosine. If catabolites are also accumulated by the heart, phosphorylation (apparent uptake) would be even lower and deamination higher than we show in table 1. However, the rates of apparent adenosine uptake that we computed agree very well with the rates of ^{14}C-adenosine incorporation into the adenine nucleotide pool of isolated perfused rat hearts reported by Namm.[21]

Simultaneous infusion of adenosine and EHNA did not inhibit apparent adenosine uptake and EHNA did not change the total purine release from normoxic or ischaemic hearts. Therefore, we conclude that EHNA did not interfere with myocardial purine transport in our experiments. Furthermore, EHNA did not disturb normoxic or ischaemic myocardial function or energy metabolism as can be concluded from our measurements of apex displacement, purine release and myocardial high energy phosphates. Our studies confirm the observation[3 20] that there is no ATP-sparing effect of ENA and no increased salvage of adenosine during ischaemia in the presence of EHNA. We found no indications of an inhibitory effect of EHNA on myocardial AMP-deaminase as was suggested elsewhere[26] for cultured cells, but was not found in *in vitro* assays of AMP-deaminase from rat polymorphonuclear leucocytes.[27]

Our results are compatible with several hypotheses. Firstly, one could assume that EHNA does not reach the myocardial compartment where adenosine is formed and deaminated, but that EHNA inhibits adenosine deaminase in a compartment to which adenosine is transported after formation (eg interstitium, endothelium). The fact that EHNA is active in experiments with isolated cultured cells[9] does not support this hypothesis. A second hypothesis is purine formation from IMP and GMP. Myocardial purine formation by way of AMP→IMP→inosine has never been disproved. On the contrary, several studies have demonstrated a rise in myocardial IMP levels during ischaemia[8 22] and especially during anoxia.[23] Our experiments have shown that at least 60% of

purines released during ischaemia is derived from AMP→adenosine. However, part of the remaining 40% is probably due to AMP-deaminase (EC 3.5.4.6) activity in the heart. A lower energy charge (as is found during ischaemia) deinhibits AMP-deaminase activity.[24] In addition, the importance of cytosolic 5′-nucleotidase has become apparent. It has been reported for this enzyme that Km values for IMP are lower than those for AMP (see ref 25), which makes it possible that any IMP formed is broken down quickly to inosine. Another possible source of myocardial purine release is breakdown of GMP to guanosine, guanine, xanthine and uric acid. This possibility has not been investigated. Recently, however, we have studied hypoxanthine incorporation into isolated rat hearts after ischaemia. We demonstrated that 25% of hypoxanthine was built into guanine nucleotides.[15] These results showed a relatively active guanine nucleotide metabolism in rat heart after ischaemia. Still, the incorporation of hypoxanthine (via IMP) into AMP is very small compared with normoxic and ischaemic purine release.[15] Therefore it does not influence purine release in our experiments where adenosine or EHNA was infused.

In conclusion, we have demonstrated that deamination is the main pathway of catabolism of exogenous adenosine in the isolated perfused rat heart, even at low (1 μmol·litre^{-1}) concentration of adenosine. Furthermore, AMP breakdown in ischaemic rat hearts proceeds for 60% via adenosine formation. Lastly, we suggest that a considerable part of purines released from the heart is derived from IMP or GMP breakdown.

The authors wish to thank Mrs M J Kanters-Stam for typing the manuscript, Mr J A J Hegge for drawing of figures and Mr P Ph de Tombe for excellent technical support. P W Achterberg was supported by the Dutch Foundation for Medical Research (FUNGO).

References

1 Berne RM. The role of adenosine in the regulation of coronary blood flow. *Circ Res* 1980;**47**:807–13.
2 Olsson RA, Snow JA, Gentry MK. Adenosine metabolism in canine myocardial reactive hyperemia. *Circ Res* 1978;**42**:358–62.
3 Foker JE, Einzig S, Wang T. Adenosine metabolism and myocardial preservation. *J Thorac Cardiovasc Surg* 1980;**80**:506–16.
4 Reibel DK, Rovetto MJ. Myocardial adenosine salvage rates and restoration of ATP content following ischemia. *Am J Physiol* 1979;**237**:H247–52.
5 Schrader J, Baumann G, Gerlach E. Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Arch* 1977;**372**:29–35.
6 Belardinelli L, Belloni FL, Rubio R, Berne RM. Atrioventricular conduction disturbances during hypoxia: Possible role of adenosine in rabbit and guinea pig heart. *Circ Res* 1980;**47**:684–91.
7 Schütz W, Schrader J, Gerlach E. Different sites of adenosine formation in the heart. *Am J Physiol* 1981;**240**:H963–70.
8 Deuticke B, Gerlach E. Abbau freier Nucleotide in Herz, Skeletmuskel, Gehirn und Leber der Ratte bei sauerstoffmangel. *Pflügers Arch* 1966;**229**:239–54.
9 Newby AC. The interaction of inhibitors with adenosine metabolising enzymes in intact isolated cells. *Biochem Pharmacol* 1981;**30**:2611–5.
10 Rubio R, Berne RM, Dobson JG. Sites of adenosine production in cardiac and skeletal muscle. *Am J Physiol* 1973;**225**:938–53.
11 Thompson CI, Rubio R, Berne RM. Changes in adenosine and glycogen phosphorylase activity during the cardiac cycle. *Am J Physiol* 1980;**238**:H389–98.
12 Schoutsen B, De Jong JW, Harmsen E, De Tombe PP, Achterberg PW. Myocardial xanthine oxidase/dehydrogenase. *Biochim Biophys Acta* 1983;**62**:519–24.
13 Jacob MI, Berne RM. Metabolism of purine derivatives by the isolated cat heart. *Am J Physiol* 1966;**198**:322–6.
14 De Jong JW. Phosphorylation and deamination of adenosine by the isolated perfused rat heart. *Biochim Biophys Acta* 1972;**286**:252–9.
15 Harmsen E, De Tombe PP, De Jong JW, Achterberg PW. Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia. *Am J Physiol* 1984;**246**:H37–43.
16 Stam H, De Jong JW. Sephadex-induced reduction of coronary flow in the isolated rat heart: A model for ischemic heart disease. *J Mol Cell Cardiol* 1977;**9**:633–50.
17 Harmsen E, De Jong JW, Serruys PW. Hypoxanthine production by ischemic heart demonstrated by high pressure liquid chromatography of blood purine nucleosides and oxypurines. *Clin Chim Acta* 1981;**115**:73–84.
18 Harmsen E, De Tombe PP, De Jong JW. Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography. *J Chromatogr* 1982;**230**:131–6.
19 Ronca-Testoni S, Borghini F. Degradation of perfused adenine compounds up to uric acid in the isolated rat heart. *J Mol Cell Cardiol* 1982;**14**:177–80.
20 Humphrey SM, Seelye RN. Improved functional recovery of ischemic myocardium by suppression of adenosine catabolism. *J Thorac Cardiovasc Surg* 1982;**4**:16–22.
21 Namm DH. Myocardial nucleotide synthesis from purine bases and nucleosides. *Circ Res* 1973;**23**:686–95.
22 Parker JC, Smith EE, Jones CE. The role of nucleoside and nucleobase metabolism in myocardial adenine nucleotide regeneration after cardiac arrest. *Circ Shock* 1976; **3**:11–20.
23 Frick GP, Lowenstein JM. Studies of 5′-nucleotidase in the perfused rat heart. *J Biol Chem* 1976;**251**:6372–8.
24 Harmsen E, Verwoerd TC, Achterberg PW, De Jong JW. Regulation of procine heart and skeletal muscle AMP-deaminase by adenylate energy charge. *Comp Biochem Physiol* 1983;**75B**:1–3.
25 Schrader J. Metabolism of adenosine and sites of production in the heart. In: *Regulatory Function of Adenosine* (Berne RM, Rall TW, & Rubio R, eds.). pp. 133–146, The Hague, Nijhoff, 1983.
26 Henderson JF, Brox L, Zombor G, Hunting D, Lomax CA. Specificity of adenosine deaminase inhibitors. *Biochem Pharmacol* 1977;**26**:1967–72.
27 Newby AC. Role of adenosine deaminase, ecto-(5′-nucleotidase) and ecto-(non-specific phosphatase) in cyanide-induced adenosine monophosphate catabolism in rat polymorphonuclear leucocytes. *Biochem J* 1980;**186**:907–18.

APPENDIX PAPER

VI

Myocardial adenosine cycling rates during normoxia and under conditions of stimulated purine release

Biochem. J. (1986) 235, 13–17 (Printed in Great Britain)

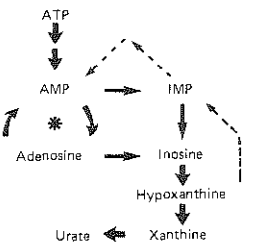
Myocardial adenosine cycling rates during normoxia and under conditions of stimulated purine release

Peter W. ACHTERBERG,* Rutger J. STROEVE and Jan Willem DE JONG
Cardiochemical Laboratory, Thoraxcentre, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

Formation and rephosphorylation of adenosine (adenosine cycling) was studied in isolated rat hearts during normoxia and under conditions of stimulated purine formation. Hearts were infused with an inhibitor of adenosine kinase (5-iodotubercidin, 2 μM). In addition, perfusions were carried out with or without acetate, which is converted into acetyl-CoA, with simultaneous breakdown of ATP to AMP and purines. We found a linear, concentration-dependent, increase in normoxic purine release by acetate (5–20 mM). Differences in total purine release with or without iodotubercidin were taken as a measure of adenosine cycling. In normoxic hearts, iodotubercidin caused a minor increase in purine release (2.7 nmol/min per g wet wt.). Acetate (12.5 mM) increased purine release by 4.9 nmol/min per g, and its combination with inhibitor gave a large increase, by 14.2 nmol/min per g. This indicates a strongly increased adenosine cycling rate during acetate infusion. However, no significant differences in purine release were observed when iodotubercidin was infused during hypoxia, anoxia or ischaemia. The hypothesis that adenosine cycling is near-maximal during normoxia was not confirmed. Increased myocardial adenosine formation appears to be regulated by the availability of AMP and not by inhibition of adenosine kinase. This enzyme mainly functions to salvage adenosine in order to prevent excessive loss of adenine nucleotides.

INTRODUCTION

Regulation of coronary blood flow by adenosine has been extensively studied (for review, see Berne, 1980). Chronotropic and dromotropic influences of adenosine on the heart have also been reported (Belardinelli *et al.*, 1980) as well as anti-catecholaminergic effects (Schrader *et al.*, 1977). Several enzymes are involved in the regulation of myocardial adenosine metabolism. Formation of adenosine can take place via 5′-nucleotidase (EC 3.1.3.5) or *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) (Schrader *et al.*, 1981; Schütz *et al.*, 1981; Achterberg *et al.*, 1985*a*). The latter enzyme is also known to bind substantial amounts of intracellular adenosine (Ueland & Saebø, 1979). Removal of adenosine occurs by adenosine deaminase (EC 3.5.4.4) or adenosine kinase (EC 2.7.1.20) and by release and washout. One hypothesis for the regulation of adenosine production and concentration (Arch & Newsholme, 1978*a*) assumes that a substrate cycle exists between adenosine and AMP (see Scheme 1) at near-maximal activity of adenosine kinase. Kinetic studies on purified adenosine kinase from rat heart (De Jong, 1977; De Jong *et al.*, 1980; Fisher & Newsholme, 1984) suggest that the above-mentioned hypothesis is feasible. However, Newby *et al.* (1983) infused the adenosine kinase inhibitor 5-iodotubercidin into isolated rat hearts and concluded from adenosine and inosine release that adenosine cycling in normoxic rat heart is virtually absent. However, in the latter study not all adenosine catabolites were determined. The present paper reports the determination of myocardial adenosine-cycling rates during normoxia and under conditions where release of myocardial purines was stimulated, e.g. low-flow ischaemia, hypoxia, anoxia or infusion of acetate during normoxia. Our data demonstrate that in normoxia the adenosine-cycling rate is low, but can be increased by acetate infusion.



Scheme 1. Metabolic pathways involved in cycling and breakdown of adenosine in the heart

* represents adenosine cycling; broken line represents hypoxanthine salvage.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade. Water (used for h.p.l.c. buffers and perfusion media) was purified with the

Abbreviation used: 5-ITu, 5-iodotubercidin (4-amino-5-iodo-7-(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine).

* To whom correspondence and reprint requests should be addressed.

Milli-Ro4/Milli-Q system (Millipore Co., Bedford, MA, U.S.A.). 5-Iodotubercidin (5-ITu; NSC 113939) was obtained through the courtesy of Dr. H. B. Wood Jr. (National Cancer Institute, Bethesda, MD, U.S.A.).

Perfusion of hearts

Male Wistar rats (275–350 g), fed *ad libitum*, were used. After anaesthesia of the rats with pentobarbital (intraperitoneally), hearts were quickly excised and immediately arrested in ice-cold 0.9% NaCl or perfusion buffer, a modified Tyrode solution, containing 10 mM-glucose (Harmsen *et al.*, 1984). The Langendorff preparation was used with 100 cmH_2O perfusion pressure. Hearts were paced at 300 beats/min (4 V stimulation for 2 ms). Perfusion temperature was kept at 37.0 ± 0.5 °C. The medium was not recirculated and was gassed with O_2/CO_2 (19:1). Hypoxia was induced by gassing the perfusion medium with $N_2/O_2/CO_2$ (13:6:1), and anoxia by gassing with N_2/CO_2 (19:1). If sodium acetate (5–20 mM) was added to the perfusate, glucose (10 mM) remained present; Na^+ was kept constant by decreasing the concentration of NaCl in the perfusion medium in accordance with the increase in sodium acetate. Ischaemia was induced by lowering perfusion pressure to 15–20 cmH_2O. At the end of the perfusion hearts were taken from the apparatus, blotted dry and weighed.

Normoxic preperfusion (15 min) preceded perfusion with modified medium (15 min) and was again followed by normoxic reperfusion (15 min).

Coronary flow

Mean coronary flow was determined by collection of perfusate in a graduated cylinder over 5 min periods. It was expressed as ml/min per g wet wt. The flow measured between 10 and 15 min preperfusion was taken as 100%.

Myocardial function

Apex displacement was used as an indicator of myocardial performance. It has been shown to correlate well with changes in myocardial pressure development during ischaemia, hypoxia and anoxia (Stam & De Jong, 1977). The displacement observed 15 min after the start of the perfusion was taken as 100%.

Assay of purines

Adenosine, inosine, hypoxanthine, xanthine and uric acid were determined by a slightly modified version of the h.p.l.c. method described by Harmsen *et al.* (1984). In brief, 200 μl of perfusate were injected on to a μBondapak C_{18} column (30 cm × 0.4 cm; Waters Associates, Milford, MA, U.S.A.) and eluted isocratically with 70 mM-KH_2PO_4, pH 4.5, to which methanol was added. Flow rate was either 1 or 3 ml/min (100 or 30 ml of methanol added to 1 litre of buffer respectively). Peak heights, detected at 254 nm, were compared with those of standards. Perfusates were collected on ice and transferred to the h.p.l.c. equipment, where temperature was kept below 5 °C.

Statistics

Results are presented as means ± S.E.M. Statistical significance was evaluated by using Student's *t* test for paired or unpaired observations, and *P* values were calculated. $P \geq 0.05$ was considered not significant.

RESULTS

Normoxia

Purine release from isolated normoxic rat hearts decreased gradually from about 5.5 nmol/min per g after 15 min preperfusion to 2–3 nmol/min per g at the end of the 45 min perfusion period. When the adenosine kinase inhibitor 5-ITu (2 μM) was infused, the basic purine release was increased by 2.7 nmol/min per g ($P < 0.05$). Acetate (5–20 mM) was infused because it can increase intramyocardial AMP and purine release, which amounted to 4.9 nmol/min per g at 12.5 mM-acetate. The combination of acetate and 5-ITu, however, caused a very strong increase in myocardial purine release (by 14.2 nmol/min per g), which was significantly greater ($P < 0.05$) than the combined increases that were caused by acetate and 5-ITu alone. This reflects increased adenosine kinase activity (adenosine cycling) during acetate infusion. In Fig. 1 the total purine release over the entire perfusion period is given for control hearts and hearts infused with acetate, 5-ITu, or the combination. The contribution of adenosine, inosine, hypoxanthine, xanthine and uric acid to the total release in these experiments is shown in Fig. 2. Uric acid plus xanthine contribute 60–80% to the total release and therefore also to observed increases in release. Acetate infusion (5–20 mM) gave a linear, concentration-dependent, increase in normoxic purine release (by 7 nmol/min per g at 20 mM-acetate). All increases in purine release were computed for individual hearts by subtracting the average of pre- and post-experimental control release from the release during the last 5 min of experimental infusion.

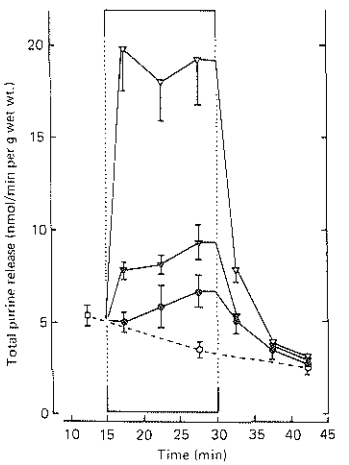


Fig. 1. Total purine release from isolated rat hearts perfused with iodotubercidin, acetate or both

Purine release (nmol/min per g wet wt.) is the average of total release over 5 min periods. From 15 to 30 min, acetate, iodotubercidin or both were infused. ○, Controls; □, combined controls; ▼, 12.5 mM-acetate; ●, 5-ITu (2 μM); ▽, acetate plus 5-ITu. Vertical bars indicate S.E.M. for four to six experiments.

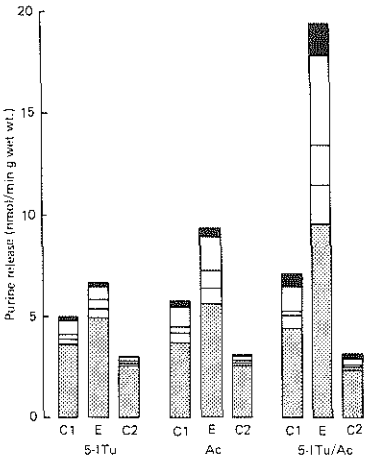


Fig. 2. Composition of total purine release during perfusion with iodotubercidin, acetate or both

Release of uric acid, xanthine, hypoxanthine, inosine and adenosine (subdivision of bars from bottom upwards) is given at the end of preperfusion (C1), at the end of reperfusion (C2) and during the last 5 min of infusion of acetate (Ac), 5-ITu or the combination (E) (see also the legend to Fig. 1 and the Materials and methods section).

Acetate caused a concentration-dependent increase in coronary flow, ranging from 130 to 165% of control flow. Infusion of 5-ITu gave a very strong vasodilation, both in the presence of acetate (220% of control flow; $P < 0.05$) and when given alone (240% of controls; $P < 0.05$). Apex displacement, which was used as an index of myocardial contractility, was not significantly changed by infusion of acetate with or without 5-ITu.

Hypoxia, anoxia, ischaemia

Perfusion of hearts with medium gassed with $O_2/N_2/CO_2$ (6:13:1) (hypoxia) caused an increase in normoxic purine release (to 9 nmol/min per g). No significant differences in purine release were observed when 5-ITu (2 μM) was present during hypoxia (Fig. 3*a*). Coronary flow increased to 130–140% of control values ($P < 0.05$) and contractility decreased to 85% of controls $P < 0.05$).

When hearts were made anoxic (N_2/CO_2, 19:1), a very strong increase in purine release was observed (to 72 nmol/min per g). The presence of 5-ITu gave a non-significant decrease in purine release as compared with anoxia alone (to 61 nmol/min per g) (Fig. 3*c*). The only observable effect of 5-ITu on anoxic purine release is the fact that the relative contribution of adenosine to total purine release is higher (28 versus 22% of total; $P < 0.05$) when 5-ITu is infused.

Anoxia caused a significant increase in coronary flow to 150% of controls ($P < 0.05$) and a strong decline in apex displacement (to 15%; $P < 0.05$) at the end of anoxia. Reoxygenation (15 min) gave a 70% return of contractility. No differences in flow or contractility were

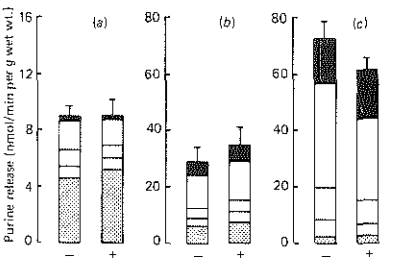


Fig. 3. Influence of iodotubercidin on myocardial purine release during hypoxia (*a*), ischaemia (*b*) and anoxia (*c*)

Total purine release during the last 5 min of hypoxic (30% O_2), ischaemic or anoxic (0% O_2) perfusion was determined in the presence (+) or absence (–) of 5-ITu (2 μM). Subdivisions of bars represent (from bottom up): uric acid, xanthine, hypoxanthine, inosine, adenosine. Vertical bars indicate S.E.M. for three to six experiments. Note the difference in scale between hypoxia and the others.

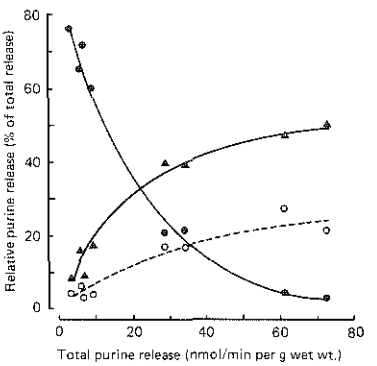


Fig. 4. Contribution of uric acid, inosine and adenosine to total purine release under various conditions

The release of uric acid (●), inosine (▲) and adenosine (○) is plotted as a percentage of total purine release under conditions of varying purine release (i.e. normoxia, hypoxia, anoxia, ischaemia). Each point is the average of three to six experiments.

observed in the presence of 5-ITu during anoxia or hypoxia.

Decreasing the perfusion pressure to 20–25% of control resulted in a strongly decreased coronary flow (to 6–8% of control) and a purine release of 30 nmol/min per g. Infusion of 5-ITu caused a non-significant increase in ischaemic purine release, to 35 nmol/min per g (Fig. 3*b*). In both groups apex displacement declined to 22.4 ± 13.4% of controls ($P < 0.05$). Apex displacement tended to be higher (non-significantly) when 5-ITu was infused during anoxia and ischaemia.

The relative contribution of the various purines to total purine release changed gradually when the total purine

release was increased (Fig. 4). The contribution of uric acid decreased from 70% (normoxic release) to less than 5% (anoxia) and the contribution of inosine increased from 10 to 50% (both $P < 0.05$). The relative release of adenosine increased gradually from less than 5 to about 25% of total purine release ($P < 0.05$).

DISCUSSION

Newby *et al.* (1983) reported that isolated normoxic rat hearts perfused with the adenosine kinase inhibitor 5-ITu do not release increased amounts of adenosine and inosine. It was hypothesized, however (Arch & Newsholme, 1978*a*; Fisher & Newsholme, 1984), that basic adenosine cycling takes place in the heart at near-maximal adenosine kinase activity. This activity is reported to be in the range of 100–200 nmol/min per g wet wt. in rat heart homogenates (Arch & Newsholme, 1978*b*) and to be about 30–40 nmol/min per g when adenosine is infused into isolated rat hearts (Aussedat *et al.*, 1984; Newby *et al.*, 1983). It was therefore expected that inhibition of adenosine kinase during normoxia should lead to highly increased formation and release of adenosine and its catabolites. However, because of very rapid breakdown of adenosine, a major part of purines that are released from adult normoxic rat hearts consists of uric acid and xanthine (Ronca-Testoni & Borghini, 1982; Schoutsen *et al.*, 1983; Achterberg *et al.*, 1985*a*,*b*). The reported absence of an effect of 5-ITu on release of adenosine and inosine might therefore be explained by assuming that the expected increase would be expressed mainly in the release of xanthine and uric acid. In our hands, infusion of 5-ITu (2 μM) into normoxic rat hearts indeed gave a small but significant increase (2.7 nmol/min per g wet wt.) in total purine release, of which 60–80% could be accounted for by xanthine and uric acid. The efficacy of 5-ITu as inhibitor of adenosine kinase in this experimental set-up is essential. It was shown (Newby *et al.*, 1983) that 5-ITu (1 μM) inhibits the incorporation of [^{14}C]adenosine (10 μM) into the myocardial adenine nucleotide pool by more than 90% and also that 5-ITu accumulated intracellularly. It still remained to be demonstrated that 5-ITu infusion could indeed be used to measure adenosine cycling, for instance when cycling in normoxic hearts takes place at a higher rate. With this in mind, acetate (5–20 mM) was infused into normoxic hearts, and a concentration-dependent increase in purine release was found. Acetate is readily taken up by the heart (Taegtmeyer *et al.*, 1980) and transformed into acetyl-CoA. In the course of this reaction, AMP is formed from ATP. Elevation of intracellular AMP content by acetate has been described for isolated rat heart (Williamson, 1965) and for dog muscle *in vivo* (Liang & Lowenstein, 1978). The latter authors also report elevation of coronary-sinus purine release and coronary vasodilation during acetate infusion. The hypothesis that adenosine-cycling rate is normally high also predicts a decrease in cycling rate when adenosine formation is increased. However, simultaneous infusion of acetate and 5-ITu gave a much greater increase in purine release (14.2 nmol/min per g) than the sum of increases caused by acetate (4.9 nmol/min per g) and 5-ITu (2.7 nmol/min per g) alone. This clearly demonstrates increased adenosine-cycling activity in the presence of acetate. In addition, these results show that infusion of 5-ITu is useful to determine adenosine-cycling rates in isolated perfused rat hearts. No significant cycling rates could be detected, however, during hypoxia, anoxia or low-flow ischaemia. Relatively high adenosine-cycling rates (20 nmol/min per g of liver) have been reported (Bontemps *et al.*, 1983) in cultured rat hepatocytes during incubation with 5-ITu. However, these cultures continually release substantial amounts of purines. Intercellular adenosine exchange, which is not the same as adenosine cycling, can take place more easily in cell cultures than in the perfused heart, because in the latter system any adenosine formed will be more rapidly washed out. Newby *et al.* (1983) reported that incorporation of infused adenosine into isolated perfused rat heart continued at a high rate (33 nmol/min per g) during severe hypoxia. This suggests that adenosine kinase is not inhibited and that cycling could take place, if adenosine is available at the site of adenosine kinase activity. These results and ours could be explained by assuming that adenosine formation takes place at a site distant from adenosine kinase or that a major part of purine formation takes place via AMP → IMP → inosine, thereby bypassing adenosine formation (Achterberg *et al.*, 1985*a*,*b*). Evidence has been presented (Rovetto & Williams, 1983) that myocardial adenosine formation from AMP is a transmembrane process by which adenosine is formed and released at an extracellular site.

Both the maximal incorporation rates of infused adenosine (Namm, 1973; Reibel & Rovetto, 1979; Newby *et al.*, 1983; Aussedat *et al.*, 1984) and the cycling rates that we found in this study are at least 2-fold lower than the maximal adenosine kinase activities that were reported *in vitro* (Arch & Newsholme, 1978*b*; Newby *et al.*, 1983). This points either to a strong inhibition of intracellular adenosine kinase (but see Newby *et al.*, 1983) or to a rate-limiting effect of adenosine transport in incorporation studies (Bowditch *et al.*, 1985).

Significant effects of adenosine on coronary flow are observed at less than 0.1 μM-adenosine in the perfusion fluid (Schrader *et al.*, 1977). It can be computed that an increase in adenosine formation of less than 2 nmol/min per g wet wt. will be sufficient for significant coronary vasodilation. A minor shift in the adenylate kinase equilibrium (Newsholme & Start, 1973) can probably cause the increase in AMP that is needed for such a low adenosine production. During acetate infusion and hypoxia a good correlation is found between total purine release and coronary flow (results not shown). The fact that no increases in adenosine concentrations were found can be explained by the rapid breakdown of adenosine to uric acid (Achterberg *et al.*, 1985*b*).

The acetate-induced increase in purine release and coronary vasidilation that we observed is presumably adenosine-mediated and similar to the reported coronary vasodilation caused by fatty acids (Hülsmann, 1976). Acetate (20 mM) caused a 65% increase in coronary flow, but a 3–4-fold increase in purine release, which indicates that increased washout is not the cause of increased purine release.

The relative contributions of the various purines to total purine release (Fig. 4) again emphasizes the importance of measuring all catabolites in these kinds of studies.

Re-incorporation of hypoxanthine could theoretically cause an underestimation of total purine formation (see Scheme 1). However, Harmsen *et al.* (1984) have shown that the rate of hypoxanthine salvage is at least one order

of magnitude lower than normoxic purine release, even when this salvage is stimulated by infusion of ribose or after ischaemia.

It appears that the main importance of adenosine kinase is to salvage the myocardial adenine nucleotide pool (Newby, 1985). Our experiments with 5-ITu show that half of the amount of purines produced can be salvaged through adenosine kinase at low and mildly increased rates of purine production. This allows a maximal cellular signal (adenosine concentration) with a minimal waste of ATP. Our final conclusion is that adenosine production, insofar as it is relevant to coronary vasodilation, is regulated not by inhibition of adenosine kinase, but most probably by the intracellular AMP concentration. Adenosine kinase appears mainly to be involved in salvage of the myocardial ATP pool, for example during increased adenosine production caused by high rates of fatty acid activation.

We thank Mrs. M. J. Kanters-Stam for typing the manuscript. P.W.A. was supported by the Netherlands Foundation for Medical Research (FUNGO/ZWO).

REFERENCES

Achterberg, P. W., De Tombe, P. P., Harmsen, E. & De Jong, J. W. (1985*a*) Biochim. Biophys. Acta **840**, 393–400
Achterberg, P. W., Harmsen, E. & De Jong, J. W. (1985*b*) Cardiovasc. Res. **19**, 593–598
Arch, J. R. S. & Newsholme, E. A. (1978*a*) Essays Biochem. **14**, 82–123
Arch, J. R. S. & Newsholme, E. A. (1978*b*) Biochem. J. **174**, 965–977
Aussedat, J., Verdys, M. & Rossi, A. (1984) Arch. Int. Physiol. Biochim. **92**, 203–217
Belardinelli, L., Belloni, F. L., Rubio, R. & Berne, R. M. (1980) Circ. Res. **47**, 684–691
Berne, R. M. (1980) Circ. Res. **47**, 807–813
Bontemps, F., Van den Berghe, G. & Hers, H.-G. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2829–2833
Bowditch, J., Brown, A. K. & Dow, J. W. (1985) Biochim. Biophys. Acta **844**, 119–128
De Jong, J. W. (1977) Arch. Int. Physiol. Biochim. **85**, 557–569
De Jong, J. W., Keijzer, E., Uitendaal, M. P. & Harmsen, E. (1980) Anal. Biochem. **101**, 407–412
Fisher, M. N. & Newsholme, E. A. (1984) Biochem. J. **221**, 521–528
Harmsen, E., De Tombe, P. P., De Jong, J. W. & Achterberg, P. W. (1984) Am. J. Physiol. **246**, H37–H43
Hülsmann, W. C. (1976) Basic Res. Cardiol. **71**, 179–191
Liang, C.-S. & Lowenstein, J. M. (1978) J. Clin. Invest. **62**, 1029–1038
Namm, D. H. (1973) Circ. Res. **23**, 686–695
Newby, A. C. (1985) Biochem. J. **226**, 343–344
Newby, A. C., Holmquist, C. A., Illingworth, J. & Pearson, J. D. (1983) Biochem. J. **214**, 317–323
Newsholme, E. A. & Start, C. (1973) Regulation in Metabolism, pp. 111–113, John Wiley and Sons, London
Reibel, D. K. & Rovetto, M. J. (1979) Am. J. Physiol. **237**, H247–H252
Ronca-Testoni, S. & Borghini, F. (1982) J. Mol. Cell. Cardiol. **14**, 177–180
Rovetto, M. J. & Williams, D. O. (1983) in Regulatory Function of Adenosine (Berne, R. M., Rall, T. W. & Rubio, R., eds.), pp. 536–537, Martinus Nijhoff Publishers, Boston
Schoutsen, B., De Jong, J. W., Harmsen, E., De Tombe, P. P. & Achterberg, P. W. (1983) Biochim. Biophys. Acta **762**, 519–524
Schrader, J., Baumann, G. & Gerlach, E. (1977) Pflügers Arch. **372**, 29–35
Schrader, J., Schütz, W. & Bardenheuer, H. (1981) Biochem. J. **196**, 65–70
Schütz, W., Schrader, J. & Gerlach, E. (1981) Am. J. Physiol. **240**, H963–H970
Stam, H. & De Jong, J. W. (1977) J. Mol. Cell. Cardiol. **9**, 633–650
Taegtmeyer, H., Hems, R. & Krebs, H. A. (1980) Biochem. J. **186**, 701–711
Ueland, P. M. & Saebø, J. (1979) Biochim. Biophys. Acta **587**, 341–352
Williamson, J. R. (1965) J. Biol. Chem. **240**, 2308–2321

Received 4 September 1985/31 October 1985; accepted 12 November 1985