Adenine Nucleotide Metabolism in the Heart

By Robert M. Berne, and Rafael Rubio

ABSTRACT

Brief periods of myocardial ischemia or hypoxia resulted in the rapid formation of adenosine from intracellular nucleotides. Adenosine levels were also increased in cardiac hypertrophy. When hypertrophied hearts were subjected to ischemia, the rate and magnitude of adenine nucleotide degradation were enhanced, but nucleotides did not appear to escape from intact ischemic cells. 5'-Nucleotidase, the enzyme that catalyzes the conversion of adenosine monophosphate (AMP) to adenosine, was associated with the sarcolemma, transverse tubules, and intercalated discs of the myocardial cells. Hence, adenosine is formed at the cell margins, and that fraction that escapes into the interstitial fluid can act locally to dilate the coronary resistance vessels. Most of the adenosine formed probably re-enters the myocardial cells and is phosphorylated to AMP. However, adenosine that crosses the vascular endothelium undergoes further degradation to inosine and hypoxanthine by enzymes located in the vascular endothelium.

The rate of de novo synthesis of cardiac nucleotides is quite slow and is greatly accelerated by cardiac ischemia or hypoxia, but falls somewhat short of the calculated loss during control and ischemic or hypoxic conditions. In contrast to de novo synthesis, phosphorylation of nucleosides (the salvage pathway) proceeds at a rapid rate. Studies on red cell ghosts revealed that: (1) adenosine enters the cells by facilitated diffusion as well as by simple diffusion; (2) at extracellular concentrations of adenosine up to 10 μM, practically all the adenosine is phosphorylated by adenosine kinase to nucleotides; (3) inosine and hypoxanthine formation predominate at extracellular levels of adenosine above 10 μM and reach equal concentrations in cells and medium; and (4) free adenosine is not present within the ghost cells until pharmacological levels (1 mM) of adenosine are reached in the medium. The reason for the preferential phosphorylation of adenosine at low extracellular concentrations is presumably a greater affinity of adenosine kinase than of adenosine deaminase for adenosine. Similar studies with dispersed embryonic chick heart cells indicated that adenosine was incorporated into myocardial cell nucleotides, but formation of inosine and hypoxanthine was much greater than in red cell ghosts. Free adenosine was not found within the embryonic heart cells even at 1 mM concentrations of adenosine. In the isolated perfused guinea pig heart uniformly labeled adenosine was found to be directly phosphorylated by adenosine kinase to AMP without prior degradation. In contrast, inosine was first degraded to hypoxanthine prior to its incorporation into nucleotides.

These observations are in agreement with the concept that adenosine formed at the myocardial cell margins by the action of 5'-nucleotidase is either released into the interstitial fluid where it serves as a metabolic vasodilator, or it is taken up again by the myocardial cells to replenish the cellular stores of adenine nucleotides.

KEY WORDS human erythrocyte ghosts myocardial ischemia or hypoxia de novo synthesis of nucleotides salvage pathway adenosine inosine hypoxanthine 5'-nucleotidase adenosine deaminase adenosine kinase nucleoside phosphorylase coronary dilation cardiac hypertrophy dog rat guinea pig rabbit

The general pathways of adenosine triphosphate (ATP) synthesis and degradation have been known for many years, and the rate of ATP utilization in energy processes and the maintenance of adequate ATP stores under physiological and pathophysiological conditions have been extensively investi- gated. Our interest in adenine nucleotide metabolism stems from our observations that adenosine formed by dephosphorylation of adenosine monophosphate (AMP) appears to be importantly involved in the adjustments of coronary blood flow to the metabolic requirements of the heart. Furthermore, recent studies have suggested that adenosine may also play a role in the regulation of blood flow in other vascular beds such as skeletal muscle and brain. Initially, we examined the degradative
pathways of adenine nucleotides to nucleosides and purine bases, and recently investigated the formation of adenine nucleotides from nucleoside precursors.

**Degradation of Adenine Nucleotides**

Myocardial hypoxia results in the release of inosine and hypoxanthine, but not of detectable amounts of adenosine. Since these substances could arise either from dephosphorylation of AMP to adenosine and deamination of adenosine to inosine or by deamination of AMP to inosine monophosphate (IMP) with subsequent dephosphorylation to inosine, it was necessary to demonstrate that adenosine was formed in AMP degradation for the adenosine hypothesis for coronary blood flow regulation to be tenable. These studies were performed with in situ hypoxic perfused hearts and with excised ischemic rabbit hearts.

In perfused hearts subjected to hypoxia for 1 to 3 minutes, ATP decreased, adenosine diphosphate (ADP) and AMP increased, but only trace quantities of adenosine, inosine, hypoxanthine, and IMP could be detected. However, in excised cardiac tissue incubated in a moist chamber at 37°C for 1 to 20 minutes, significant increases of all these compounds occurred, with approximately equal concentrations of adenosine and IMP and considerably higher and progressively increasing levels of inosine and hypoxanthine. Hence, adenosine as well as IMP were formed during AMP degradation, but were presumably washed out of the in situ hearts by the perfusing blood. These observations on adenosine formation in ischemic myocardium were in agreement with those of Gerlach et al.

The next step in support of the adenosine hypothesis was to demonstrate that adenosine is formed and released by the beating heart. This was accomplished first in guinea pig and cat hearts perfused by the Langendorff technique with the aid of the adenosine deaminase inhibitor 8-azaguanine. With anoxia, adenosine appeared in the perfusate along with greatly increased quantities of inosine and hypoxanthine. Furthermore, the amounts of adenosine, inosine, and hypoxanthine released by the perfused cat hearts were roughly inversely proportional to the oxygen tension of the perfusion medium. Subsequent technical improvements in the quantification of adenosine and its metabolites and protection against adenosine destruction by blood permitted studies on the in situ heart of the open-chest dog. These studies revealed that with brief periods of ischemia (30 to 60 seconds), measurable quantities of adenosine were released into the coronary sinus blood. Based on the adenosine levels in coronary sinus blood, the measured rate of adenosine destruction in blood, and the assumption that because of the high adenosine deaminase activity in the myocardial cells adenosine was limited to the extracellular space, the adenosine concentration in the vicinity of the coronary resistance vessels was calculated and found to be greater than the amount of infused adenosine required to elicit vasodilation of the magnitude seen during reactive hyperemia.

If adenosine serves as the mediator of metabolic control of coronary resistance, it must also be present in the normal well-oxygenated heart. This question was studied in the open-chest dog by superfusion of the epicardial surface of the heart via tubes inserted into the pericardial sac and by collection and analysis of normal pericardial fluid.

Adenosine was found with both procedures and, assuming pericardial fluid is in equilibrium with myocardial interstitial fluid, the myocardial concentration of adenosine was calculated to be 0.22 nmole/g wet tissue. This agrees reasonably well with the value of 0.32 nmole/g myocardium reported by Olsson, who employed a more sensitive method for direct measurement of adenosine on samples of cardiac tissue.

Since it was established that adenosine is present in the normal heart and increases in concentration when the heart is made hypoxic, it is necessary to know whether the nucleoside is produced rapidly enough to be compatible with the short time course of resistance decrease that occurs with hypoxia or ischemia. To this end, rat ventricles were instantaneously frozen with tongs precooled in liquid nitrogen at 0 time and at 5-second intervals after cross clamping at the atrioventricular groove. The results are illustrated in Figure 1, and indicate that adenosine is very rapidly formed in hearts following interruption of their blood supply, but after 25 seconds of ischemia the level begins to decline as the rate of deamination of adenosine exceeds the rate of its formation.

With cardiac hypertrophy produced by exercise (swimming), thyroxine administration, or subdiaphragmatic constriction of the aorta, Dr. Degenring in our laboratory has observed a decrease in myocardial ATP, an increase in ADP and AMP, a small increase in adenosine and inosine, and no change in hypoxanthine levels (Table 1). When the hearts were excised and incubated at 37°C in a moist chamber for 5 minutes, the nucleotide changes...
MYOCARDIAL METABOLISM

![Graph](image)

**Figure 1**

Effect of ischemia on myocardial adenosine levels in the rat heart.

were more marked and the adenosine and inosine levels in the hypertrophied hearts increased significantly above those of the control hearts that were also subjected to 5 minutes of ischemia (Table 1). The reduction of ATP in the hypertrophied hearts may be due to myocardial hypoxia as a result of the greater diffusion distances that develop as the cardiac fibers greatly increase in diameter without a concomitant increase in the capillary: fiber ratio. With ischemia, the higher value for adenosine and inosine in the hypertrophied hearts may be attributable to the higher substrate (AMP) levels that prevailed in the hypertrophied hearts prior to the start of ischemia.

Since (a) adenosine appears in effluents of hypoxic hearts, (b) the amounts found are too great to be accounted for by other than myocardial cells, and (c) the myocardial cells contain high concentrations of adenosine deaminase in the cell sap, knowledge of the site of adenosine formation is important. If 5'-nucleotidase, the enzyme that converts AMP to adenosine, is distributed through-out the myocardial cell, it is difficult to understand what prevents deamination of adenosine to inosine within the cell as rapidly as adenosine is formed. However, if 5'-nucleotidase is strategically located at the cell margins, adenosine could reach the interstitial space, where it would remain intact since destructive enzymes are not present in the interstitial fluid. Localization of 5'-nucleotidase was studied in rat, guinea pig, and dog hearts by histochemical techniques and electron microscopy.

The hearts were lightly prefixed by brief coronary perfusion with dilute glutaraldehyde and dithiothreitol, cut into 40- to 50-μ sections, and incubated in a buffered solution containing Pb (NO₃)₂ and with AMP or IMP as substrate. The sections were then postfixed and prepared for electron microscopy. At sites containing 5'-nucleotidase, the phosphate split from the mononucleotide reacted with the lead to produce an electron-dense precipitate. The precipitate was limited to the sarcolemma, the intercalated discs, the transverse tubules, the flattened sarcoplastic reticulum, and the axial branches of the transverse tubules. Hence the 5'-nucleotidase is located at sites contiguous with the interstitial fluid compartment. These results are illustrated in Figure 2, where IMP (Fig. 2A) and AMP (Fig. 2B) were used as substrates in sections of rat heart, and are in agreement with those of Rostgaard and Behnke.

Other phosphate compounds, including 2'- and 3'-mononucleotides, gave no precipitate, indicating the specificity of the enzyme. Observations by Baer and Drummond that rat hearts perfused with Tyrode solution containing AMP converted a major fraction of the AMP to adenosine in one passage through the heart are consonant with the concept of localization of 5'-nucleotidase at the periphery of the myocardial cells. These findings support the idea that adenosine is formed at the cell margins and can directly enter the interstitial fluid and hence reduce the contractile state of the arteriolar smooth muscle.

The adenosine that enters the vascular compartment apparently undergoes degradation to inosine and hypoxanthine, since the ratio of adenosine to inosine or hypoxanthine is considerably greater in myocardial tissue than in the coronary effluents. This degradation could be attributable to the erythrocytes in blood-perfused hearts, but similar ratios were observed in hearts perfused with Krebs-Henseleit solution.

For guinea pig myocardium (assigning a value of 1 for adenosine), the ratio of adenosine:inosine:hypoxanthine was 1:1.2:0.6.

*Supplement III to Circulation Research, Vols. 34 and 35, September 1974*
III-112  

**TABLE 1**  

*Levels of Adenine Nucleotides and Their Derivatives in Normal and Hypertrophied Rat Myocardium*  

(μmoles/g Wet Tissue)

|                      | Controls  
|----------------------|----------  
|                      | (n = 10)  
|                      | Exercise*  
|                      | (n = 6)  
|                      | Thyroxinet†  
|                      | (n = 6)  
|                      | Aortic  
|                      | constriction‡  
|                      | (n = 9)  
| Without Ischemia     |          1.17 ± 0.09 (sd)  
|                      | Heart weight (g)  
|                      | 1.55 ± 0.10  
|                      | 1.57 ± 0.08  
|                      | 1.54 ± 0.04  
|                      | Heart wt/body wt  
|                      | 3.12  
|                      | 5.39  
|                      | 6.03  
|                      | 5.23  
|                      | (mg/g)  
|                      | ATP  
|                      | 5.433 ± 0.421  
|                      | ADP  
|                      | 1.292 ± 0.194  
|                      | AMP  
|                      | 0.212 ± 0.043  
|                      | Adenosine  
|                      | 0.008 ± 0.001  
|                      | Inosine  
|                      | 0.007 ± 0.001  
|                      | Hypoxanthine  
|                      | 0.007 ± 0.001  
|                      | TOTAL  
|                      | 6.959  
| Five Minutes of Ischemia |          1.17 ± 0.09 (sd)  
|                      | Heart weight (g)  
|                      | 2.511 ± 0.384  
|                      | 2.287 ± 0.600  
|                      | 2.312 ± 0.115  
|                      | Heart wt/body wt  
|                      | 4.015 ± 0.291  
|                      | 2.511 ± 0.210  
|                      | 2.056 ± 0.306  
|                      | 1.937 ± 0.120  
|                      | (mg/g)  
|                      | ATP  
|                      | 1.518 ± 0.079  
|                      | ADP  
|                      | 1.007 ± 0.132  
|                      | AMP  
|                      | 0.073 ± 0.004  
|                      | Adenosine  
|                      | 0.068 ± 0.006  
|                      | Inosine  
|                      | 0.037 ± 0.007  
|                      | TOTAL  
|                      | 6.411  
|                      | 5.708  
|                      | 5.564  

* Two to three half-hour periods of swimming per day for seven weeks.  
† After seven weeks' treatment with D-thyroxine.  
‡ Nine days after operation with subdiaphragmatic constriction of the aorta.

whereas the ratio in guinea pig cardiac perfusates was 1:7:6. Adenosine deaminase is a ubiquitous enzyme, and we know of no histochemical method for determining its location in tissue. However, the conversion of inosine to hypoxanthine is a reversible reaction catalyzed by the enzyme nucleoside phosphorylase (inosine + inorganic phosphate = hypoxanthine + ribose-1-P0₄), and essentially the same technique used for the localization of 5'-nucleotidase was employed in the determination of sites of action of nucleoside phosphorylase. After light prefixation, sections of rat, guinea pig, or dog heart were incubated in a buffered solution containing hypoxanthine and ribose-1-P0₄ and the liberated phosphate was trapped with lead. The tissues were then prepared for electron microscopy. As shown in Figure 3, the lead phosphate precipitate which indicates the sites of nucleoside phosphorylase activity was limited to the endothelial cells, pericytes, and erythrocytes. Note that no evidence of nucleoside phosphorylase activity is visible in the myocardial cells. These observations indicate that as adenosine crosses the capillary endothelium it is converted to inosine and hypoxanthine and accounts for the different ratios of adenosine to its degradative products in myocardium and coronary effluents. In blood-perfused hearts the concentrations of inosine and hypoxanthine relative to adenosine are even greater due to further adenosine degradation by the formed elements of the blood. Recent studies in liver and kidney revealed a similar distribution of nucleoside phosphorylase.

In these isolated tissues perfused with Krebs-Henseleit solution one can collect and analyze the tissue secretions, as well as the venous effluent and tissue, for adenosine, inosine, and hypoxanthine. Data from experiments on guinea pig liver and kidney are shown in Table 2, and it is evident that ratios of adenosine to inosine and hypoxanthine are greater in bile and urine than in the venous effluent from liver and kidney, respectively. This suggests that the enzymes that degrade adenosine are more active in vascular endothelium than in the tubular structures that carry away the secretions of these two tissues. In support of this suggestion are
Distribution of S' nucleotidase in the rat heart, indicated by the dense particles of lead phosphate. 

A: with 1 mm IMP as substrate; B: with 1 mm AMP as substrate. SC = subsarcolemmal cisterna; ISF = interstitial fluid; ID = intercalated disc; TT = transverse tubule; FSR = flattened sarcoplasmic reticulum; Ax-T = axial-directed branch of transverse tubule.

Supplement III to Circulation Research, Vols. 34 and 35, September 1974
histochemical findings that nucleoside phosphorylase is primarily located in the vascular endothelium of guinea pig liver and kidney. To what extent these observations apply to other tissues remains to be determined.

**Synthesis of Adenine Nucleotides**

Since there is a continuous but small loss of adenosine and its degradative products from the normal heart, and this loss is enhanced when the oxygen supply becomes inadequate for the cardiac needs, and since the adenine nucleotide pool of the myocardium is held fairly constant, it is apparent that nucleotide synthesis keeps pace with degradation. Purine nucleotide synthesis can occur by *de novo* synthesis from nonpurine precursors (for example, glycine), by condensation of a purine base (adenine or hypoxanthine) with phosphoribosylpyrophosphate (PRPP), or by phosphorylation of a purine nucleoside (adenosine or inosine). In extracts of pig heart, Goldthwait demonstrated that purine nucleotide synthesis occurred by all three pathways and that *de novo* synthesis was the least active. Recently, Zimmer et al. studied *de novo* synthesis of adenine nucleotides in rat hearts in situ and in isolated perfused rat hearts using labeled glycine as substrate. The rate of *de novo* synthesis in the intact heart was comparable to the values obtained with pig heart extracts and is approximately equal to the calculated loss of hypoxanthine. When the in situ hearts of open-chest rats were subjected to hypoxia by brief repetitive interruptions of respiration, the rate of *de novo* synthesis was increased 100%, whereas the isolated perfused hearts showed a 580% increase following 20 minutes of ischemia. However, the control values of *de novo* synthesis in the isolated perfused hearts was only about one-sixth that of the in situ

**TABLE 2**

<table>
<thead>
<tr>
<th>Effluent</th>
<th>Hyp (nmol/ml)</th>
<th>Ado (nmol/ml)</th>
<th>Ino (nmol/ml)</th>
<th>Hyp:Ado:Ino</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venous</td>
<td>0.35</td>
<td>0.00</td>
<td>0.14</td>
<td>266:1:1:10</td>
</tr>
<tr>
<td>Biliary</td>
<td>0.33</td>
<td>0.06</td>
<td>0.23</td>
<td>5.38:1:3.77</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venous</td>
<td>0.38</td>
<td>0.06</td>
<td>0.26</td>
<td>6.7:1:4.7</td>
</tr>
<tr>
<td>Ureteral</td>
<td>2.02</td>
<td>3.41</td>
<td>2.43</td>
<td>0.6:1:0.7</td>
</tr>
</tbody>
</table>

Abbreviations: Hyp = hypoxanthine; Ado = adenosine; Ino = inosine.

_Supplement III to Circulation Research, Vols. 34 and 35, September 1974_
hearts. Although the increased rate of de novo synthesis resulting from an insufficient oxygen supply tended to compensate for the loss of nucleotide incurred during hypoxia, the rate was too slow to account for the recovery of myocardial nucleotide stores by this route alone. Hence, synthesis from purines or nucleosides must play an important role in replenishing or maintaining myocardial adenine nucleotides. In support of this proposal are the findings of Isselhard et al.\textsuperscript{18} that in the anesthetized rabbit, restoration of adenine nucleotide stores following 4 successive periods of 1 to 3 minutes of asphyxia was not significantly influenced by intravenous infusion of purine-ring precursors, adenine, or inosine, but was greatly accelerated by infusions of adenosine. In cardiac hypertrophy produced in rats by constriction of the aorta, de novo synthesis of adenine nucleotides from labeled glycine was significantly increased in the first to the fifth days after the constriction.\textsuperscript{19}

Jacob and Berne\textsuperscript{20} studied the uptake of labeled adenosine in the isolated perfused cat heart and found that in one passage through the coronary vascular bed about 57% of the adenosine was taken up by the heart and incorporated into adenine nucleotides. Similar rates of labeled adenosine incorporation were observed with the anoxic perfused cat heart.\textsuperscript{17} This degree of incorporation suggested that adenosine is directly phosphorylated to AMP and that uptake is not significantly altered by anoxic conditions that would be expected to result in a net loss of myocardial adenine nucleotides. However, these studies did not provide proof of direct phosphorylation, nor did they indicate the mechanism of adenosine uptake and incorporation.

Since the intact heart is a complex structure consisting of vessels, nerves, and fibrous tissue as well as myocardial cells, and has at least three compartments (intracellular, interstitial, and intravascular), a simple model was selected for a study of adenosine uptake and metabolism. The model used was the human red cell ghost.\textsuperscript{13} After hemolysis, the cells were reconstituted by addition of salt in the presence of ATP and Mg\textsuperscript{++} and placed in suspension medium to form a two-compartment system separated by a membrane containing active enzymes. After incubation with different concentrations of uniformly labeled adenosine in the medium, the reaction could be stopped by rapid cooling and the cells and medium separated and analyzed separately for nucleotides, nucleosides, and bases. The uptake curve of adenosine by the ghost cells showed two components: a rapid, non-linear rate at extracellular concentrations of 1 to 10 $\mu$M, which suggested a carrier-mediated mechanism (facilitated diffusion); and a slower, linear rate of uptake at extracellular concentrations above 10 $\mu$M (simple diffusion). When the ghost cells were incubated in 5 $\mu$M adenosine for increasing periods of time up to 30 minutes, the adenosine disappeared, inosine and hypoxanthine appeared in approximately equal concentrations in cells and medium, and labeled nucleotides appeared only within the cells, indicating that the ghost cells are readily permeable to adenosine, inosine, and hypoxanthine, but not to nucleotides.

Analysis of labeled nucleotides within the ghost cells and of degradation products of adenosine in cells and medium indicated that incorporation into nucleotides reached a plateau at about 10 $\mu$M extracellular adenosine concentrations and that at higher adenosine levels the nucleoside was almost totally converted to inosine and hypoxanthine (Fig. 4). These findings suggest that the adenosine kinase (enzyme that catalyzes phosphorylation of adenosine to AMP) saturates at 10 $\mu$M adenosine, whereas adenosine deaminase failed to saturate at levels of adenosine as high as 50 $\mu$M. The reason for the selective phosphorylation of adenosine at low extracellular concentrations is probably attributable to the fact that the $K_m$ of myocardial adenosine kinase\textsuperscript{24} is significantly lower than that of myocardial.

![Graph showing incorporation of adenosine into red cell ghosts and its intracellular metabolism to inosine, hypoxanthine, and adenine nucleotides.](image-url)

*FIGURE 4*  
Incorporation rates of adenosine into red cell ghosts and its intracellular metabolism to inosine, hypoxanthine, and adenine nucleotides. Data were calculated from uptake values obtained during 5 minutes of incubation at 37°C. Each point represents the average of 2 experiments. (Reprinted from the American Journal of Physiology,\textsuperscript{13} by permission.)
dial adenosine deaminase, and hence has a greater affinity for the adenosine.

One of the assumptions on which the adenosine hypothesis for regulation of coronary blood flow was based is that free adenosine does not exist within the cells because of the high intracellular adenosine deaminase concentration. To test this assumption, extracellular adenosine was increased to pharmacological levels (1 mM), and only at this concentration was any free adenosine detected within the ghost cells. Uptake of labeled adenosine was shown by Olsson and co-workers to follow Michaelis-Menten kinetics when infused into the left coronary artery of the open-chest dog. The Km value was greater and the Vmax value less for adenosine uptake than for dog heart adenosine kinase, suggesting that penetration of the cell by adenosine was the rate-limiting step in nucleotide formation. Furthermore, an adenosine analogue that inhibits facilitated diffusion of nucleosides in erythrocytes inhibited adenosine uptake by the dog heart, providing further evidence of a carrier-mediated process of adenosine uptake. Finally, dipyridamole, which blocks adenosine uptake by cells, including erythrocyte ghosts, failed to inhibit adenosine kinase activity.

To study adenosine uptake and metabolism by myocardial cells and to avoid the complexities of intact heart (nonmyocardial cells and three compartments), dispersed embryonic chick heart cells suspended in cell culture medium were employed. The technique was quite similar to that used for the red cell ghosts. Incubation of cells from 12-day-old embryos in medium containing labeled adenosine resulted in incorporation of the radioactive into intracellular nucleotides, the formation of large amounts of intracellular and extracellular inosine and small amounts of intracellular and extracellular hypoxanthine, and the appearance of AMP in the medium. When hearts of embryos of 16 days or older were used, the nucleotides were all confined to the cells. Control experiments with washed 12-day-old embryonic heart cells containing prelabeled nucleotides showed no leakage of AMP into the medium. Hence, it appears that myocardial cells of young embryos have adenosine kinase activity at the cell periphery and that with maturation this enzyme activity at the external surface of the cell disappears.

The striking differences between the dispersed embryonic heart cells and the erythrocyte ghosts were a greater deamination to inosine by the cardiac cells and a lesser degree of incorporation of the labeled adenosine into intracellular nucleotides. A similarity between these two preparations was that free adenosine was not present within the embryonic heart cells at extracellular concentrations of adenosine as high as 1 mM (Table 3). In cultured, beating, rat heart cells, the addition of adenosine to the culture medium produced a 50% increase in intracellular ATP associated with an increase in the beating rate, and suggests a possible metabolic regulatory role for adenosine.

On the basis of previous work, it had been proposed that adenosine incorporation into adenine nucleotides occurred by direct phosphorylation by adenosine kinase. This concept was supported by Liu and Feinberg, who found that in the isolated perfused rabbit heart adenosine was incorporated without loss of the ribose moiety, since adenosine was not detected, and without loss of the amino group, since adenosine incorporation exceeded inosine incorporation 15- to 20-fold. However, Maguire et al., using extracts of rat heart, found adenosine deaminase activity to be more than 2-fold greater than adenosine kinase activity, and concluded that adenosine is first degraded to hypoxanthine prior to incorporation into nucleotides. This difference in results may be due to the difference between intact tissue and extracts or to species differences, since Hopkins and Goldie found that rat hearts take up less adenosine than guinea pig hearts, and that uptake is blocked by dipyridamole in guinea pig but not in rat heart. The authors suggest that perhaps rat heart lacks membrane-bound adenosine kinase.

A direct approach to the problems of the mechanism of adenosine incorporation into nucleotides was undertaken by Wiedmeier et al. Isolated guinea pig hearts were perfused with oxygenated Krebs-Henseleit solution containing uniformly labeled adenosine at concentrations that did not alter coronary flow. Perfusates were collected for 8 minutes of perfusion; the hearts were then rapidly

### TABLE 3

Percentage of Total Adenosine Uptake in Chick Embryonic Heart Cells

<table>
<thead>
<tr>
<th>Conc. adenosine in medium (μM)</th>
<th>Nucleotides</th>
<th>Inosine</th>
<th>Hypoxanthine</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>6.2</td>
<td>92.3</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>1,000</td>
<td>24.6</td>
<td>63.0</td>
<td>12.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Supplement III to Circulation Research, Vols. 34 and 35, September 1974*
### Table 4

<table>
<thead>
<tr>
<th>Substance hydrolyzed</th>
<th>Nucleoside moiety (inosine)</th>
<th>Base moiety (hypoxanthine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts/ min</td>
<td>Amount measured (nmoles)</td>
</tr>
<tr>
<td>Adenosine from perfusion fluid</td>
<td>581,020</td>
<td>3.54</td>
</tr>
<tr>
<td>Adenine nucleotides from heart perfused with adenosine</td>
<td>1</td>
<td>1,274</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>664</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,454</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>835</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,266</td>
</tr>
<tr>
<td>Average (88)</td>
<td></td>
<td>1.43 ± 0.03</td>
</tr>
</tbody>
</table>

| Inosine from perfusion fluid                        | 1,527,560    | 1.56                      | 984,104                | 1,216,675    | 2.51                      | 485,890                | 2.03               |
| Adenine nucleotides from heart perfused with inosine | 1            | 278                       | 380                    | 0.73         | 174                       | 205                    | 0.85               | 0.86               |
|                                                     | 2            | 184                       | 195                    | 0.94         | 132                       | 160                    | 0.83               | 1.13               |
|                                                     | 3            | 228                       | 320                    | 0.71         | 110                       | 170                    | 0.65               | 1.09               |
|                                                     | 4            | 200                       | 315                    | 0.63         | 90                        | 175                    | 0.51               | 1.24               |
|                                                     | 5            | 310                       | 305                    | 1.02         | 142                       | 200                    | 0.71               | 1.44               |
| Average (88)                                        |              | 1.15 ± 0.10               |

*Inosine-U-14C was prepared from a different batch of adenosine-U-14C than used in the adenosine perfusion experiments; hence the difference in specific activity (SA) ratios of nucleoside-to-base for the two nucleosides in the perfusion fluid.*

Frozen between aluminum blocks precooled in liquid nitrogen. Perfusates and tissue were analyzed for nucleotides, nucleosides, and bases, and the tissue nucleotides were enzymatically degraded to inosine and hypoxanthine. The specific activity (SA) of the inosine and hypoxanthine obtained from the degraded nucleotides was determined, as was the specific activity of the infused adenosine. If the ratio of specific activity of inosine to hypoxanthine from the nucleotides is equal to the ratio of specific activity of inosine to hypoxanthine of the infused adenosine that was also enzymatically degraded to inosine and hypoxanthine, it would indicate that the adenosine was directly phosphorylated to AMP. If the infused adenosine were first split to inosine and hypoxanthine prior to incorporation into nucleotides, then the ratio of inosine to hypoxanthine would approach a value of 1, since the previously labeled ribose moiety of the nucleoside would be replaced from the large pool of unlabeled ribose.

From Table 4 it can be seen that the ratio of specific activity of the nucleoside to base of the degraded cardiac nucleotides did not differ significantly from that of the perfused adenosine. However, when uniformly labeled inosine was added to the perfusion fluid and the samples handled in the same way as for adenosine perfusion, the ratios for the cardiac nucleotides were not significantly different from 1.0, and were significantly different from the ratio of the perfused inosine (Table 4). These findings indicate that adenosine is directly incorporated into adenine nucleotides without prior degradation, whereas inosine is first split to hypoxanthine and ribose-1-PO₄ and the base is then incorporated into the adenine nucleotides.

Although considerable information has been gained about adenine nucleotide degradation and synthesis, more specific quantification is needed, particularly during different physiological and pathophysiological states.

**References**

Discussion

Dr. A. M. Vikhert, Moscow, U.S.S.R.: It is especially interesting that the hypertrophied heart exhibits increased formation of mononucleotides and adenosine. The hypertrophied heart, when associated with increased arterial pressure, is extremely sensitive to ischemia, as evidenced by the fact that sudden death is much higher in this group than in a similar group with normal blood pressure.
This has been studied extensively in the Soviet Union and shown to be the case. Possibly the mechanism for this difference is related to the nucleotides. In your study hypertrophy was produced by three different mechanisms, one being physiological, the other two pathological. Was the reaction to ischemia the same regardless of the mechanism used to produce hypertrophy?

Dr. Berne: The three different methods used yielded essentially the same results with respect to the degree of hypertrophy, the ratio of heart to body weight, and the magnitude of the change in nucleotides. Dr. Meerson has shown, as has Dr. Wearn, that the fiber-to-capillary ratio remains the same in hypertrophy, but the fiber diameter increases markedly. I suspect, but have no proof, that the increased fiber diameter is a limiting factor for the diffusion of oxygen which, in turn, causes increased degradation of nucleotides.

Dr. L. F. Nikolaeva, Moscow, U.S.S.R.: With electron microscopy we have demonstrated myocardial changes following the injection of inosine; but this will better be discussed following my presentation.

Dr. A. M. Chernukh, Moscow, U.S.S.R.: Dr. Berne, you have clearly demonstrated that coronary flow is coupled to metabolism via the effect of adenosine as an arteriolar vasodilator. Your diagram indicated that adenosine has some sort of influence on both the endothelium and the pericytes. There is now a viewpoint that pericytes can change capillary permeability. Do you agree with that, or did I misunderstand you?

Dr. Berne: Well, I don't think adenosine per se has an influence on the endothelium. What I was trying to show was that the enzyme nucleoside phosphorylase is limited to the endothelium and the pericytes and that this is the site of adenosine degradation to inosine and hypoxanthine.

It is interesting that you mentioned permeability, in that many years ago Dr. Gerlach did some studies on other organs, as well as the heart, with respect to degradation of nucleotides. In skeletal muscle it was felt that AMP was first deaminated to inosinic acid and then to inosine and hypoxanthine without the formation of adenosine. This we now know to be incorrect, because with better methods we can detect adenosine. However, in the brain, even though methods were crude, he demonstrated that this tissue formed adenosine during degradation of nucleotides. Buyinski and Rapela injected adenosine into the cerebral arterial circulation and found that adenosine has essentially no vasodilator effect on cerebral vessels. Later it occurred to us that perhaps the adenosine was not getting across the blood-brain barrier. Recently, we injected adenosine into the carotid artery of a dog, and it had essentially no effect on pial vessel diameter determined by direct observation. However, when adenosine was applied directly to the pial vessels, it proved to be a very potent dilator. Furthermore, if adenosine is infused into a carotid artery, very little incorporation of the radioactivity into brain nucleotides is obtained. However, if the nucleoside is placed in the cerebrospinal fluid, there is rapid formation of labeled nucleotides. We did experiments with hypoxic brain tissue and cerebrospinal fluid and found adenosine and its breakdown products to be rapidly released into the cerebrospinal fluid, but the adenosine did not cross the blood-brain barrier to any significant extent. Thus, there is apparently conservation of nucleotides in the nervous system, since the nucleosides released into the cerebrospinal fluid are taken up again by the cerebral tissue.

Dr. Steven E. Mayer, La Jolla, California: Have you considered the possibility that adenosine may be formed as the result of degradation of cyclic AMP? I realize that at first this doesn't sound likely because the cyclic AMP concentration in heart is probably only about one-fiftieth of the adenosine concentration. Nevertheless, the concentration of cyclic AMP at any one time reflects the rate of synthesis and degradation. Also, there is a large accumulation of cyclic AMP during hypoxia, at least in heart and brain.

Dr. Berne: Yes, we have considered it for the very reason that you mentioned. I do not think it is a strong possibility, but it may be a contributing factor. It is of some interest that adenosine greatly enhances the concentration of cyclic AMP in the brain and that vascular smooth muscle relaxation is associated with high concentrations of cyclic AMP. We are now trying to determine whether adenosine will induce changes in the concentration of cyclic AMP in vascular smooth muscle which, in turn, might induce vasodilation.

Dr. Eugene Braunwald, Boston, Massachusetts: What is your opinion of the concept that the response of vascular smooth muscle to adenosine is conditioned by the pO2? There is also the possibility that prostaglandins mediate coronary vasodilation that occurs in reactive hyperemia. I mention the latter because Dr. Theodore Cooper's group has shown recently that indomethacin, which blocks the synthesis of prostaglandins, also significantly reduces reactive hyperemia following a brief period of coronary occlusion.
Dr. Berne: With respect to the first question, I presume you are referring primarily to the work of Detar and his colleagues, who showed that varying the O₂ tension had little effect on helical strips of coronary arteries in vitro. However, if small amounts of adenosine were present, the vessels relaxed, with very small decreases in oxygen tension. Detar postulated that the adenosine concentration remains constant and that the oxygen tension is the regulator of coronary vascular resistance. I must take issue with this concept for the simple reason that our observations show that the adenosine levels are not constant but change proportionately with the changes in coronary flow and inversely with changes in oxygen tension. There may be some synergism between oxygen tension and adenosine, but I do not think pO₂ is the primary factor. However, I hasten to add that many local factors may be involved, such as pH, K⁺, and pCO₂. Another argument against oxygen tension as the primary regulator of coronary blood flow is that during reactive hyperemia that occurs after release of a coronary artery after 10, 20, or 30 seconds of occlusion, coronary sinus blood draining the heart is bright red (oxyhemoglobin). Since the venous blood is practically saturated with oxygen, so must be the capillary blood and the immediate environment of the arterioles. Therefore, one would expect constriction rather than dilation shortly after release of an occluded coronary artery. Furthermore, there is a good correlation, within limits, between duration of the occlusion and duration of the reactive hyperemia. These observations cannot be explained on the basis of oxygen tension.

With respect to your question about the prosta
glandins, this is a highly controversial point, and I regret that Dr. Cooper had to leave before this topic was brought up for discussion. Dr. Cooper's group presented data which showed that indomethacin caused a decrease in the reactive hyperemic response and a decrease in the release of prostaglandin. At the same meeting a group from Michigan State presented a paper showing that indomethacin was completely without effect on reactive hyperemia. Work in our laboratory showed that indomethacin did not affect the hyperemic response in the heart, whereas it was effective in the kidneys. It is also of interest that Logan and Wiedmeier found that administration of prostaglandin E₁ to the heart resulted in an increase in release of adenosine from the myocardium.

Dr. Braunwald: Dr. Vatner, in his work on prostaglandins, observed a minor effect of indomethacin on reactive hyperemia in the coronary circulation, and a profound effect in the renal circulation.

Dr. Ruth Hegyeli, Bethesda, Maryland: Have you observed the same embryonic heart cells as they grow in tissue culture at different stages of interaction? A fantastic network develops between them within one to two days, and I was wondering if the response is the same before and after contact?

Dr. Berne: We have not done that type of experiment. We have been working mostly with dispersed embryonic chick heart cells, but plan to use a preparation of cultured heart cells from newborn rats, since Dr. Seraydarian has shown that the addition of adenosine to these cultured cells enhances their activity and raises their ATP levels. We were rather surprised to find very low levels of ATP in the dispersed cells and in the cultured cells. We did make one observation that is rather interesting and that disturbed us for a long time. If 12-day-old embryonic chick heart cells are incubated with labeled adenosine, a lot of labeled AMP is found in the medium as well as in the cells. We thought we had leaky cells. However, labeled nucleotides did not appear in the medium with incubation of dispersed cells containing labeled nucleotides. Hence, the cell membranes remained intact. When the experiments were done with embryos 16 to 18 days old, labeled nucleotides did not appear in the medium. Thus, it is apparent that as you have said, the cells are continuously undergoing changes. We expect that these young cells have adenosine kinase present on the external surface of the membrane and that this is lost as the cells mature. This change takes place somewhere around the 15th day of embryonic life.
Adenine Nucleotide Metabolism in the Heart
R. M. Beme and R. Rubio

doi: 10.1161/01.RES.35.3_suppl.III-109

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/35/3_suppl/III-109.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/