Adenine Nucleotide Metabolism and Compartmentalization in Isolated Adult Rat Heart Cells

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SUMMARY. The metabolism and intracellular compartmentalization of adenine nucleotides in a preparation of adult rat heart myocytes showing good morphology, viability, and tolerance to calcium ion has been examined by high performance liquid chromatography. These myocytes contain an average of 23 nmol adenine nucleotide per milligram protein which is about 60% of the adenine nucleotide content of intact rat heart tissue. The loss of adenine nucleotide occurs during the incubation and washing steps that increase the yield of viable cells, rather than during the collagenase perfusion. An analysis of cellular compartments shows that the adenine nucleotide of the cell consists of 17 nmol adenine nucleotide in the cytosol, 5 nmol in the mitochondria, and 1.3 nmol adenosine diphosphate bound to myofibrils per milligram cell protein. Myocytes lose both adenosine triphosphate and adenine nucleotide when incubated anaerobically in the absence of glucose, and the lost adenine nucleotide can be accounted for as increased inosine, adenosine, and inosine monophosphate. Myocytes that contain less than 0.1 nmol of cytosol adenosine triphosphate per milligram cell protein maintain an intact sarcolemma, but are unable to carry out anaerobic glycolysis. Reoxygenation of anaerobic cells results in restoration of energy charge and a net resynthesis of about 2 nmol adenine nucleotide per milligram protein. Adenosine and inosine monophosphate decrease on reoxygenation of anaerobic cells, whereas inosine levels increase. When iodoacetate is added to block glycolysis, the decline in adenine nucleotide and production of inosine monophosphate are accelerated and there is no resynthesis of adenine nucleotide when anaerobic cells are reoxygenated. Large accumulations of inosine monophosphate are also seen in myocytes treated with an uncoupler of oxidative phosphorylation. (Circ Res 54: 536-546, 1984)

MYOCARDIAL ischemia is characterized by the depletion of cellular high-energy phosphates and a parallel decline in total adenine nucleotides (AN) (see Jennings et al., 1981, for example). The decrease in AN is reflected in increased levels of inosine, hypoxanthine, and xanthine. These analyses are consistent with the degradation of AN by conversion of AMP to adenosine by 5'-nucleotidase (see Berne, 1980, for a review) and subsequent conversion of adenosine to inosine by adenosine deaminase. Further degradation of inosine to hypoxanthine appears to occur in vascular cells, but not in myocytes (Berne, 1980). Heart muscle has been shown to contain an active purine nucleotide cycle (Takala et al., 1980), but little IMP has been found in ischemic hearts (Jennings et al., 1981; Swain et al., 1982). For this reason, the alternative degradation sequence involving the conversion of AMP to IMP by AMP deaminase (Baer et al., 1966), followed by conversion of IMP to inosine, has not been considered a major pathway for AN degradation.

Preparations of isolated adult ventricular cells are now available in which the morphological and metabolic features of intact heart tissue are well-preserved (Dow et al., 1981; Hohl et al., 1982; Kao et al., 1980; Cheung et al., 1982, for example). Such myocyte preparations would appear to offer a convenient route to the study of nucleotide synthesis and degradation under conditions existing in the myocardial cell, as well as to the intracellular compartmentalization of these components. Myocytes lose ATP and AN as a function of time of anaerobic incubation in the absence of glucose (Hohl et al., 1982). In the present study, a sensitive anion exchange HPLC (high performance liquid chromatography) method was employed to establish the products that result from ATP and AN degradation. The distribution of these components to AN resynthesis. The distribution of these components between the cytosol, mitochondrial, and myofibrillar fractions of the cell was also determined. It is concluded that isolated myocytes degrade AN to adenosine and inosine and that IMP accumulation can be quite large under conditions of rapid and complete depletion of high-energy phosphates.
Methods

Myocytes were isolated from hearts of adult male rats by collagenase perfusion, as recently described (Hohl et al., 1983). Morphology was routinely examined by light microscopy, and viability was determined by trypan blue exclusion (Altschuld et al., 1981b) or retention of lactate dehydrogenase (Murphy et al., 1982). Metabolite data are presented as nmol/mg biuret protein (Gornall et al., 1949). Cells were washed and incubated in a Krebs-Ringer phosphate buffer (pH 7.2) containing NaCl (120 mm), KCl (6 mm), KH2PO4 (1.2 mm), Na2HPO4 (16 mm), MgCl2 (1.2 mm), creatine (20 mm), tauroine (60 mm), bovine serum albumin (BSA, 2%), and an amino acid and vitamin supplement (see Hohl et al., 1983, for details). Where indicated, glucose (11 mm) and Ca++ (1 mm) were present. Incubations were carried out either anaerobically or aerobically in this medium, as previously described (Altschuld et al., 1981b). Rates of glycolysis were estimated as described by Hohl et al. (1983). Total cell water was determined by 2H2O distribution and corrected for extracellular water by [14C]polyethylene glycol distribution (Hohl et al., 1983). The cell preparations used in this study contained more than 90% viable cells (by trypan blue exclusion), and more than 85% had the elongated, rod-like appearance of heart cells in situ (see Hohl et al., 1982, for a summary of the properties of these cells).

Extraction and Fractionation of Cells

Cells were simultaneously separated from the suspending medium and extracted with perchloric acid by rapid centrifugation through an organic isolation layer, essentially as described by McCune and Harris (1979) for hepatocytes. Tubes were prepared by layering 0.35 ml bromododecane over 0.1 ml 2 M perchloric acid and centrifugation for 1 minute in an Eppendorf microcentrifuge. An aqueous layer (0.5 ml of 0.25 M sucrose containing 20 mM MOPS buffer, pH 8.0, and 2 mM Tris-EGTA) was added above the bromododecane. The cell suspension (0.5 ml) then was injected into this aqueous layer and centrifuged immediately for 1–2 minutes to separate and extract the cells. The recovery of cell protein in the perchloric acid extract in this procedure was 92 ± 6% (means ± SD, n = 10). Mg++ was estimated by atomic absorption spectroscopy, using dilutions of the perchloric acid extract.

We prepared and extracted digitonin-lysed cells (Altschuld et al., 1981a) by adding 0.6 mg/ml digitonin to the aqueous layer in the above procedure. To minimize loss and metabolic alteration of the nucleotide pools, the medium was supplemented with an inhibitor-stop of atracyloside (0.24 mg/ml) and oligomycin (0.02 mg/ml). The cell suspension was injected into this aqueous layer and centrifuged immediately for 1–2 minutes to separate and extract the cells. The recovery of an internal standard (CDP) added to the 2 M perchloric acid in this procedure averaged 102 ± 9% (n = 6).

Chromatography–Nucleotides

Anion exchange HPLC of myocyte nucleotides was based on a method described by Edelson et al. (1979). The neutralized extract (250 μl) was placed on a Whatman PXS 10/25 SAX column and eluted at a flow rate of 2 ml/min with buffer A (0.005 M KH2PO4, pH 4.5) and a 1%/min gradient of buffer B (0.75 M KH2PO4, pH 4.5) for 20 minutes. This was followed by 2% buffer B/min for 17.5 minutes, and, finally, 55% buffer B for 5 minutes. For analysis requiring better separation of peaks in the early part of the program, we allowed a period of 10 minutes before initiating the gradient of B (schedule b). Chromatographic grade KH2PO4 (Matheson, Coleman, and Bell) was further purified and recrystallized following the method of Shmukler (1970).

A typical chromatogram from freshly isolated myocytes is reproduced in Figure 1. Peaks were identified by comparison of their retention times with those of authentic standards and by cochromatography with added standards. Quantification was based on peak area using an Altex CR2-A integrating recorder and external standards. Acid-labile reduced pyridine nucleotides were estimated essentially as described by Heldt et al. (1965). A known

![Figure 1. Typical HPLC nucleotide profile for rat heart myocytes.](http://circres.ahajournals.org/)
quantity of NADH was reacted with perchloric acid, and the resulting mixture was carried through the same extraction and chromatography sequence as described above. The peak labeled DF-NADH in Figure 1 cochromatographs with adenosine and inosine, but these components were readily detected in the aqueous suspending medium following separation of the cells as just described. The nucleosides were adsorbed to a boronate column and separated from nucleotides and other contaminants prior to elution with 0.1 M formic acid and quantification by HPLC (Trewyn et al., 1982).

Whole Heart Metabolites

Adult male rats were decapitated and the chest cavity was quickly opened and filled with an ice slush of Krebs-Ringer-phosphate buffer, pH 7.2. The hearts were removed and approximately 10 ml of the ice buffer was forced through the aortic stump to remove blood. The hearts were quickly freeze-clamped, ground to fine powder under liquid nitrogen, extracted with perchloric acid, and the neutralized extract was analyzed by HPLC. Three additional hearts were incubated at 37°C in a zip-lock plastic bag for 30 minutes prior to freeze clamping.

Results

Nucleotide Profile of Rat Heart

The anion exchange HPLC used in this study effectively separates the major nucleotides found in rat ventricle (see Fig. 1). Fresh rat hearts, freeze-clamped immediately after a rapid perfusion to remove blood, contain 39 nmol AN/mg protein (Table 1) when analyzed by this procedure. A portion of the cellular ATP is converted to ADP and AMP by this manipulation, since the calculated energy charge of the myocytes as isolated was 0.91 (calculated from the data of Table 1). When quantification of AMP and IMP was necessary, a slightly modified elution (schedule b) was applied when quantification of AMP and IMP was necessary. The energy charge of the myocytes as isolated was 0.91 (calculated from the data of Table 1).

Retention and Loss of Nucleotides during Myocyte Preparation

The preparation of myocytes from rat hearts involves an extended perfusion with collagenase [45–65 minutes of aerobic perfusion with glucose (Hohl et al., 1983)]. Analysis of minced heart tissue just after this collagenase perfusion shows only slight (ca. 10% or less) declines in AN and other nucleotides at this stage (Table 2). There is little change in the ratio of ATP to ADP after this collagenase perfusion shows only slight (ca. 10% or less) declines in AN and other nucleotides at this stage (Table 2) relative to intact heart tissue (Table 1). More significant losses of AN occur during the incubation and washing steps in which the number of viable cells with rod-cell morphology is markedly increased and cellular debris is removed. In the analyses shown in Table 2, the AN content after incubation with Ca++ in a shaking water bath and filtration of the disassociated cells through gauze has fallen to about 65% of that of an intact heart. The subsequent washing steps increase the percentage of viable cells in the preparation to 92% and do not result in further loss of AN or ATP (Table 2). There is little change in the ratio of ATP to ADP in the myocytes at any stage in the preparation (Table 2).

Compartmentalization of Myocyte Nucleotides

The sarcolemma of rat heart myocytes can be disrupted with digitonin under conditions that release cytosolic enzymes and metabolites but leave the mitochondrial fraction intact (Altschuld et al., 1981a; Murphy et al., 1982). Digitonin does not release AN from isolated heart mitochondria (data not shown). Analysis of digitonin-lysed myocytes shows that 6 nmol AN and about 3.3 nmol NAD(H)/mg are sedimented with the digitonin-insoluble residue (Table 1). The difference between these values and those for intact cells gives an estimate of cytosolic nucleotides of 16 nmol ATP, 17 nmol AN, and 1.3 nmol NAD(H)/mg (Table 3).

The digitonin-insoluble residue contains myo-
**TABLE 1**

Nucleotide Content of Intact Rat Heart Ventricle vs. Rat Heart Myocytes

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Fresh heart</th>
<th>Ischemic heart</th>
<th>Isolated myocytes</th>
<th>Digitonin-extracted myocytes</th>
<th>Triton-extracted myocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
<td>AMP</td>
<td>AN</td>
<td>GTP</td>
</tr>
<tr>
<td></td>
<td>24.6 ± 2.5</td>
<td>11.3 ± 2.3</td>
<td>3.1 ± 0.9</td>
<td>39.0 ± 1.4</td>
<td>0.7 ± 0.2</td>
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<td></td>
<td>24.6 ± 2.5</td>
<td>11.3 ± 2.3</td>
<td>3.1 ± 0.9</td>
<td>39.0 ± 1.4</td>
<td>0.7 ± 0.2</td>
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<td>24.6 ± 2.5</td>
<td>11.3 ± 2.3</td>
<td>3.1 ± 0.9</td>
<td>39.0 ± 1.4</td>
<td>0.7 ± 0.2</td>
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<td>24.6 ± 2.5</td>
<td>11.3 ± 2.3</td>
<td>3.1 ± 0.9</td>
<td>39.0 ± 1.4</td>
<td>0.7 ± 0.2</td>
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<td>1.5 ± 0.6</td>
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<td>14.6 ± 1.3</td>
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<td>18.3 ± 1.8</td>
<td>23.1 ± 0.6</td>
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<td>0.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.1 ± 0.1</td>
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<td>0.3 ± 0.2</td>
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<tr>
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<td>1.8 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
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<td>5.7 ± 0.1</td>
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<td>3.7 ± 0.1</td>
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<td></td>
<td>36.7 ± 1.7</td>
<td>29.9 ± 1.3</td>
<td>5.2 ± 0.3</td>
<td>74 ± 1</td>
<td>74 ± 1</td>
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<tr>
<td></td>
<td>5.2 ± 0.3</td>
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<tr>
<td></td>
<td>25.4 ± 4.5</td>
<td>21.3 ± 4.2</td>
<td>6.6 ± 0.6</td>
<td>71 ± 4</td>
<td>71 ± 4</td>
</tr>
<tr>
<td></td>
<td>26.2 ± 1.3</td>
<td>21.5 ± 1.6</td>
<td>6.1 ± 0.9</td>
<td>88 ± 2</td>
<td>88 ± 2</td>
</tr>
<tr>
<td></td>
<td>24.5 ± 0.2</td>
<td>20.0 ± 0.6</td>
<td>6.5 ± 0.8</td>
<td>92 ± 1</td>
<td>92 ± 1</td>
</tr>
</tbody>
</table>

Fresh rat hearts were extracted as described in the text. Ischemic hearts were incubated for 30 minutes at 37°C under N2, as described, and then extracted. Freshly isolated myocytes, digitonin-extracted and Triton-extracted myocytes were isolated and acid extracts prepared as described in the text. Values presented are means ± SD for duplicate determinations of nucleotides (see Fig. 1) from three hearts in each group, and from four myocyte preparations. Where no value is indicated, the nucleotide was below the detection limits of the analysis. These limits vary with the retention time and sharpness of the individual peaks, but in most instances are <0.2 nmol/mg.

**TABLE 2**

Retention and Loss of Nucleotides during the Preparation of Myocytes

<table>
<thead>
<tr>
<th>Step in preparation</th>
<th>AN</th>
<th>ATP</th>
<th>ATP:ADP</th>
<th>% Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minced tissue after perfusion with collagenase</td>
<td>36.7 ± 1.7</td>
<td>29.9 ± 1.3</td>
<td>5.2 ± 0.3</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>Mince incubated with Ca** and filtered through gauze</td>
<td>25.4 ± 4.5</td>
<td>21.3 ± 4.2</td>
<td>6.6 ± 0.6</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Cells after washing 2X in 0.5% BSA</td>
<td>26.2 ± 1.3</td>
<td>21.5 ± 1.6</td>
<td>6.1 ± 0.9</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>Final preparation</td>
<td>24.5 ± 0.2</td>
<td>20.0 ± 0.6</td>
<td>6.5 ± 0.8</td>
<td>92 ± 1</td>
</tr>
</tbody>
</table>

The details of the myocyte preparation are given in Hohl et al. (1983). Values tabulated are the means ± SD of four duplicate extractions at the indicated point in the preparation. Viability was estimated by trypan blue exclusion, as previously described (Altschuld et al., 1981b).
was permeable to \( ^{14} \text{C} \)-polyethylene glycol. This high molecular weight probe measures extracellular water in the pellet and also can be presumed to penetrate the 10% of the cells present that do not have an intact sarcolemma. We can therefore estimate that 1.76 \( \mu l/mg \) protein was present within the viable cells. For the protein of nonviable cells, this would give a value of about 1.9 \( \mu l \) water/mg protein for the viable cells. The digitonin-insoluble residue of these cells contains 0.50 \( \pm 0.16 \) (\( n = 6 \)) \( \mu l \) of polyethylene glycol-impermeable water which probably can be equated with the mitochondrial matrix water volume (Altschuld et al., 1981a). This analysis suggests that about 26% of the cellular water is mitochondrial matrix water. Using these estimates of cytosol and matrix water, the 16 \( \mu mol \) ATP/mg of the cytosol would be equivalent to 11 \( \mu mol \) ATP, whereas the 3.7 \( \mu mol \) ATP/mg in the matrix would represent 7.4 \( \mu mol \) ATP.

### Loss of Nucleotides on Anaerobic Incubation

Aerobic myocyte preparations supplemented with glucose retain ATP (and AN) well, when incubated for extended periods (Hohl et al., 1982). From 70 to 80% of the initial ATP content is maintained over a 1-hour incubation period at 37°C, and the presence or absence of 1 \( \mu M \) Ca\(^{2+}\) has little effect (data not shown). The loss of ATP from these aerobic cells is accounted for almost exclusively by a decrease in cytosol ATP, and GTP cannot be detected in myocytes after 30 minutes of aerobic incubation (not shown).

Hohl et al. (1982) reported a progressive loss of AN, as well as ATP, as a function of time of incubation of myocytes under anaerobic conditions in the absence of glucose. An analysis of cellular compartments shows that there is very little ATP in the cytosol of cells incubated for 30 minutes at 37°C in the absence of glucose or \( O_2 \) (Table 3). The ATP content of the digitonin-insoluble residue is very nearly the same as that for untreated anaerobic myocytes, so that, by difference, the ATP level of the cytosol averages 0.05 \( \mu mol/mg \) cell protein (Table 3). Of the 3.7 \( \mu mol \) AN/mg protein retained by the anaerobic cells, 1.1 \( \mu mol/mg \) is ADP bound to the myofibrils and another 1.5 \( \mu mol/mg \) is mitochondrial AN (Table 3).

It should be noted that 80% of the anaerobic cells exclude trypan blue and retain lactic dehydrogenase at this point (Table 3; Hohl et al., 1982), so that the sarcolemma of most cells appears to remain intact in the face of these very low levels of cytosolic ATP. A loss of NAD(H) to less than 50% of control also accompanies loss of AN under these conditions of de-energized incubation (Table 3). Extensive loss of both AN and NAD(H) from the mitochondrial fraction has also occurred under these conditions. Anaerobic incubation in the absence of glucose results in increased levels of IMP and, in contrast to the situation with fresh cells, significant amounts of IMP are present in the cytosol of these de-energized cells (Table 3). There is a marked increase in both adenosine and inosine in the suspending medium under these conditions (Fig. 2). The production of adenosine by anaerobic myocytes is biphasic, with a rapid initial rate of formation followed by a slower phase. Inosine formation is more linear with time of incubation (Table 3).

Since ATP is required for both hexokinase and phosphofructokinase activity, the low cytosolic ATP content of anaerobic, glucose-deprived myocytes (Table 3) may provide a rationale for the observation that these cells do not produce lactate from glucose added anaerobically (Hohl et al., 1982). Cells incu-

### TABLE 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Fresh myocytes</th>
<th>Anaerobic myocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Cytosol</td>
</tr>
<tr>
<td>ATP</td>
<td>19.5 ± 0.7</td>
<td>16.0 ± 0.6</td>
</tr>
<tr>
<td>ADP</td>
<td>3.2 ± 0.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>AMP</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>AN</td>
<td>23.2 ± 0.6</td>
<td>17.0 ± 0.4</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>4.6 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>IMP</td>
<td>1.6 ± 0.9</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Mg**</td>
<td>36 ± 1</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>

Values for cytosol nucleotides (as \( \mu mol/mg \) cell protein) were calculated from the data of Table 1 by subtracting the values for the digitonin-insoluble residue from those for intact myocytes. Mitochondrial nucleotides are those for the digitonin-extracted cells, less the nucleotide retained by Triton-extracted cells [myofibrils + cytoskeleton (see Table 1)]. Anaerobic myocytes were incubated for 30 min at 37°C without glucose in a \( N_2 \) atmosphere prior to extraction. The Triton-extracted anaerobic cells contained 1.1 \( ± 0.05 \) \( \mu mol/ADP/mg \) protein and no other detectable nucleotides. Values tabulated are means \( ± SE \) for four cell preparations. The fresh myocytes were 92 ± 1% (mean \( ± SE \)) viable and the anaerobic cells 80 ± 2% viable by the criterion of trypan blue exclusion.
Inosine

FIGURE 2. Increase in adenosine and inosine in the suspending medium of anaerobic myocytes incubated in the absence of glucose. Average of four determinations with SD indicated.

bated with 11 mM glucose produce lactate at a rate of 29 ± 3 nmol/min per mg protein (mean ± se, n = 6) throughout a 60-minute anaerobic incubation (see Fig. 2 of Hohl et al., 1983). When myocytes are incubated anaerobically in the absence of glucose to deplete cytosol ATP (see Table 3) and then challenged with 11 mM glucose (and rotenone to prevent any mitochondrial ATP production), the observed rate of lactate production is only 2 ± 2 nmol lactate/min per mg (n = 5). If these same anaerobic cells are re-oxygenated for 2 minutes before the addition of glucose and rotenone, the ATP levels are restored to the extent permitted by the available AN pool (Hohl et al., 1982), and the rate of lactate production by the nonrespiring cells is restored to 25 ± 4 nmol/min per mg (n = 4).

It is of interest that myocyte Mg ++ levels decline much less than either AN or NAD(H) during anaerobic incubation without glucose. There is a net decline of 5 nmol Mg ++/mg from fresh cells to those incubated anaerobically for 30 minutes (Table 3). Most of the Mg ++ lost is from the cytosol (7 nmol/mg).

Acceleration of ATP and AN Loss by Iodoacetate

Myocytes incubated anaerobically in the absence of glucose maintain ATP and AN levels by glyco-
genolysis for several minutes at 37°C (Hohl et al., 1982). Iodoacetate (5 mM) completely abolishes the production of lactate under these conditions and markedly accelerates the loss of cellular ATP (Fig. 3) and a parallel loss of AN (not shown). In the presence of this inhibitor, the ATP level falls to 2 nmol/mg after 5 minutes and to nearly undetectable levels as the incubation is continued (Fig. 3). The loss of AN in iodoacetate-treated myocytes is accompanied by large increases in cellular IMP (Fig. 3) and in extracellular adenosine and inosine. Myocyte IMP is maximal at 5 minutes of incubation with iodoacetate and declines thereafter, whereas inosine production proceeds at a nearly linear rate over a 30-minute incubation period.

The production of large amounts of IMP in iodoacetate-treated myocytes is somewhat unexpected, in view of the low levels of this nucleotide found in ischemic rat heart tissue (Table 1). Accumulation of IMP in rat heart myocytes is not restricted to iodoacetate-treated cells, however. The study shown in Figure 3 establishes that IMP also accumulates to significant levels in anaerobic myocytes and to high levels (6 nmol/mg) in myocytes treated with the uncoupler of oxidative phosphorylation, m-chlorocarbonyl cyanide phenylhydrazone (CCP). An analysis of the time course of nucleotide and nucleoside alterations in uncoupled myocytes is shown in Figure 4. After 2 minutes of incubation,
ATP decreases from over 16 to 2 nmol/mg. AMP rises to 5, and IMP to 4.5 nmol/mg. In the period from 2 to 5 minutes of incubation, the AMP, adenosine, and ADP start to decline while inosine increases and IMP reaches a peak of 6 nmol/mg protein (Fig. 4).

**Nucleotide Profile of Myocytes after an Anaerobic-to-Aerobic Transition**

It has been established that myocytes, incubated anaerobically without glucose to deplete ATP and AN levels, are capable of restoring the adenylate energy charge and 70% of the initial creatine phosphate level when re-oxygenated (Hohl et al., 1982). The total amount of ATP restored is limited, to a large extent, by the size of the AN pool available. The study shown in Table 4 establishes that myocytes incubated for 15 minutes in the absence of glucose and O$_2$ have depleted ATP and AN levels and increased IMP, adenosine, and inosine. Continued anaerobic incubation for an additional 15 minutes produces a decline in cellular IMP, further loss of ATP and AN, and increased inosine production (Table 4). Reaeration of these cells for 15 minutes, however, results in resynthesis of ATP from AMP and ADP and, also, a net increase in AN of about 2 nmol/mg. This is accompanied by a loss of 2.3 nmol/mg IMP, a decrease (2.2 nmol/mg) of adenosine, and marked increase in inosine (Table 4). Since the sum of AN, IMP, and the nucleosides is nearly constant in such experiments, it can be concluded that there is little degradation of nucleosides to bases, and no major conversion of these components to other nucleotides.

In the presence of iodoacetate, re-oxygenation of anaerobic myocytes results in a somewhat accelerated rate of removal of cellular IMP (relative to continued anaerobiosis), but in no net increase in AN (Table 4). In the experiments reported, IMP declined by 5.7 nmol/mg during re-oxygenation (as compared to 2.8 nmol/mg lost during the same period of anaerobiosis). Total AN decreased by 0.7 nmol/mg during this period and inosine levels continued to increase (Table 4).

**Discussion**

**AN and ATP Content of Myocytes**

The analytical procedures used in this study yield values of AN for rat heart (39 nmol/mg protein, Table 1) that are consistent with estimates obtained by enzymic procedures and by $^{31}$P-NMR. For example, Vary et al. (1979) reported an AN of 32 nmol/mg (calculated from $\mu$mol/g dry weight, using a factor of 1.2 $\mu$g dry per $\mu$g protein), the same value found by Ingwall (1982) for ATP in rat heart. It is clear that our preparations of myocytes (Hohl et al., 1983), containing 23 and 19 nmol/mg protein AN and ATP, respectively, are depleted of these components relative to intact rat hearts (ca. 60% retention of AN). These values can be corrected upward to account for the fact that 10% of the cells are nonviable and only 92% of cell protein is recovered in the bromododecane step. The corrected AN content of 28 nmol AN/mg protein represents a 72% retention of AN relative to intact tissue.

The analysis of nucleotide loss at various stages of the myocyte preparation (Table 2) is of interest, since it shows that AN is not lost during the prolonged perfusion with collagenase. The hearts soften and decrease resistance to perfusion during this process (Hohl et al., 1983), so that some loss due to local ischemia might have been expected. However, most of the AN loss occurs during the incubation of minced tissue to complete the disaggregation process (Table 2). Subsequent washing steps increase the percentage of viable cells in the final preparation, but have little effect on AN content. The fact that the ATP:ADP ratio remains relatively high and does not change throughout the preparation suggests that the cells are not becoming deenergized during any
TABLE 4

<table>
<thead>
<tr>
<th></th>
<th>15 min N2</th>
<th>30 min N2</th>
<th>15-min N2</th>
<th>15-min O2</th>
<th>15-min O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No iodoacetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>3.4 ± 0.7</td>
<td>1.6 ± 0.3*</td>
<td>-2.1</td>
<td>6.7 ± 0.6*</td>
<td>+3.3</td>
</tr>
<tr>
<td>AN</td>
<td>6.7 ± 0.6</td>
<td>4.2 ± 0.4*</td>
<td>-2.8</td>
<td>8.6 ± 0.4*</td>
<td>+1.9</td>
</tr>
<tr>
<td>IMP</td>
<td>3.0 ± 0.3</td>
<td>3.2 ± 0.4</td>
<td>+0.2</td>
<td>0.7 ± 0.2*</td>
<td>-2.3</td>
</tr>
<tr>
<td>Adenosine</td>
<td>5.4 ± 0.9</td>
<td>5.2 ± 0.4</td>
<td>-0.2</td>
<td>3.2 ± 0.2*</td>
<td>-2.2</td>
</tr>
<tr>
<td>Inosine</td>
<td>6.4 ± 1.0</td>
<td>11.2 ± 0.8*</td>
<td>+4.8</td>
<td>10.9 ± 1.6*</td>
<td>+4.5</td>
</tr>
<tr>
<td>Σ</td>
<td>21.8</td>
<td>23.8</td>
<td>23.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodoacetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>1.0 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>-0.4</td>
<td>0.9 ± 0.3</td>
<td>-0.1</td>
</tr>
<tr>
<td>AN</td>
<td>3.5 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>-1.1</td>
<td>2.8 ± 0.3</td>
<td>-0.7</td>
</tr>
<tr>
<td>IMP</td>
<td>7.0 ± 0.6</td>
<td>4.2 ± 0.3</td>
<td>-2.8</td>
<td>1.3 ± 0.2</td>
<td>-5.7</td>
</tr>
<tr>
<td>Adenosine</td>
<td>4.3 ± 0.4</td>
<td>4.4 ± 0.9</td>
<td>+0.1</td>
<td>2.6 ± 0.1*</td>
<td>-1.7</td>
</tr>
<tr>
<td>Inosine</td>
<td>9.5 ± 0.7</td>
<td>13.6 ± 1.3*</td>
<td>+4.1</td>
<td>13.2 ± 0.5*</td>
<td>+3.7</td>
</tr>
<tr>
<td>Σ</td>
<td>24.3</td>
<td>24.6</td>
<td>19.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Myocytes were incubated under N2 in the absence of glucose for 15 minutes (±5 mM iodoacetate). At this point, half of the cells were reoxygenated and both the anaerobic and oxygenated cells were incubated for an additional 15 minutes. Values tabulated are means ± SE of four to six such experiments. The Δ values represent the increase or decrease from the corresponding value at 15 minutes of incubation. Σ is the sum of AN, IMP, and the nucleoside values.

*P < 0.05 vs. 15-minutes N2.

of these manipulations. The final preparation used in the present work and in the study of Hohl et al. (1982) contained over 90% viable cells with an average ATP of 19 nmol/mg protein. This preparation appears quite comparable to that of Kao et al. (1980) who reported 95% viability and ATP of 20 nmol/mg protein. Two recent preparations that report higher ATP content, Cheung et al. (1982) and Montini et al. (1981) (27 and 25 nmol/mg protein, respectively) contain significantly fewer viable cells (>70% and 77% viable, respectively). The basis for this difference is not clear, but it is possible that either a less rigorous removal of nonviable components results in AN preservation, or the presence of nonviable cells promotes the restoration of AN levels in viable cells. It is noteworthy that Piper et al. (1982) were able to plate out rod-shaped myocytes with removal of nonviable cells. These preparations, which are presumed to consist entirely of viable rod cells, have AN and ATP levels that are equal to or greater than the 39 nmol AN/mg protein of intact rat heart tissue (Piper et al., 1982). In this case, the cells may be able to resynthesize AN lost during the cell preparation, once they become attached and are supplied with components present in a tissue culture medium, since they can be maintained in this condition for extended periods of time.

Compartmentalization of AN in Myocytes

These studies establish that 6 nmol AN out of 23 nmol/mg protein sediment with the digitonin-insoluble residue (Table 1), so that 17 nmol AN/mg can be considered to be cytosol nucleotides. The value for cytosol ADP of 1.0 nmol/mg cell protein (Table 3) is considerably higher than the corresponding value (0.2 to 0.3 nmol/mg protein) calculated from the creatine phosphokinase equilibrium (see Steenbergen et al., 1978, for example). Some error is inherent in the estimation of this value by subtraction as is done in Table 3, but it is also likely that a portion of the ADP released by digitonin is bound to cytosol proteins as has been found in the case of hepatocytes (Gankema et al., 1983). Treatment of isolated mitochondria with digitonin at the concentrations used to disrupt the sarcolemma of myocytes does not release mitochondrial AN (100 ± 2% recovery of AN in three determinations). However, it is possible that digitonin does release some ADP bound to other membranous components in the digitonin-insoluble residue, and that this ADP will contribute to that calculated as cytosol nucleotide by this procedure.

The present treatment does appear to eliminate the contribution of ADP contained in F-actin polymers that is liberated by perchloric acid and which contributes to erroneously high estimates of cytosol ADP in direct analysis of heart muscle (see Seraydarian et al., 1962). Triton extraction of myocytes removes all traces of mitochondria, sarcoplasmic reticulum, and other lipid-containing membranes (Brierley et al., 1983; Altschuld et al., 1983) and the Triton-insoluble fraction contains 1.3 nmol ADP/mg cell protein (Table 1). This value corresponds well to previous estimates of actin-bound ADP in
rat heart (Kohn et al., 1977). To obtain a valid estimate of mitochondrial AN, a correction must be applied to the AN analysis of the digitonin-insoluble residue to account for this actin-bound ADP. With this correction, the mitochondrial AN amounts to 4.9 nmol/mg, or 21% of cellular AN. When the nucleotide analysis is combined with estimates of intracellular water distribution, ATP concentrations of 11 mM for the cytosol and 7.4 mM for the matrix are obtained. These values are in good agreement with those reported by Soboll and Bunger (1981) who used non-aqueous fractionation of freeze-clamped guinea pig hearts to establish values of 10 mM for cytosol and 5.6 mM for mitochondrial ATP. However, with a similar approach, Kauppinen et al. (1980) obtained values of 8.1 and 6.0 mM for cytosolic ATP in beating vs. arrested rat hearts. The corresponding mitochondrial values were 6.0 and 2.8 mM, respectively (Kauppinen et al., 1980). We should note that discrepancies have been found when the mitochondrial ATP:ADP ratio is estimated by non-aqueous fractionation in hepatocytes as opposed to intact perfused liver (Soboll et al., 1980), and that non-aqueous fractionation does not account for myofibrillar ADP directly.

It has been estimated by morphometric analysis that the mitochondrial volume is 35% of the total cell volume (Smith and Page, 1976). Our value of 0.5 μl/mg cell protein (26% of cell water) for mitochondrial matrix water is compatible with these volume estimates, since the 3H2O value does not account for the volume occupied by the membranes and the intermembrane space of the mitochondria. Estimates of the percentage of cellular protein that account for the volume occupied by the membranes and the intermembrane space of the mitochondria. Estimates of the percentage of cellular protein that account for the volume occupied by the membranes and the intermembrane space of the mitochondria. Estimates of the percentage of cellular protein that account for the volume occupied by the membranes and the intermembrane space of the mitochondria. Estimates of the percentage of cellular protein that account for the volume occupied by the membranes and the intermembrane space of the mitochondria. Estimates of the percentage of cellular protein that account for the volume occupied by the membranes and the intermembrane space of the mitochondria.

These studies and those of Hohl et al. (1982) show that cytosol ATP is lost to very low levels in anaerobic myocytes, and that virtually all cellular ATP is mitochondrial after about 20 minutes of anaerobic incubation. Mitochondrial ATP is increased on reaeration (Hohl et al., 1982), but it is not yet clear whether AN can be elevated in this fraction in short-term incubations. Both AN and NAD(H) are lost to a greater extent than Mg2+ from the mitochondria in deenergized myocytes (Table 3). This and the corresponding retention of much of the cytosol Mg2+ in anaerobic myocytes suggests that specific degradation pathways are involved in the nucleotide loss, rather than generalized increases in sarcolemmal and mitochondrial permeability.

It should be emphasized that the low levels of cytosol ATP do not produce sarcolemmal damage in anaerobic myocytes (Table 3) and that large numbers of viable cells remain in these preparations for extended periods with essentially no ATP in the cytosol (see Hohl et al., 1982, for viability curves). These results appear to conflict with earlier conclusions based on analysis of intact hearts (Gebhard et al., 1977) and cultured neonatal heart cells (Altona and Van der Laarse, 1982) in which loss of sarcolemmal integrity appears directly related to energy deprivation.

Pathways for AN Degradation and Resynthesis

The degradation of the AN pool of anaerobic myocytes results when ATP hydrolysis to ADP exceeds the rate of its regeneration by glycolysis. Under these conditions, AMP is produced by adenylate kinase and converted to adenosine by 5'-nucleotidase or to IMP by AMP deaminase (Fig. 5). There is considerable evidence (Schutz et al., 1982) that the 5'-nucleotidase responsible for the production of adenosine in hypoxic cells is cytosolic and has properties that differ from those of the exo-enzyme of the sarcolemma that is thought to deliver adenosine to the interstitial space for action as a vasodilator (see Berne, 1980). The adenosine produced in the myocyte cytosol is transported out of the cells (Schutz et al., 1982). Isolated myocytes do not appear to degrade inosine, since long-term incubations (Fig. 4, for example) show complete purine recovery as nucleotides and nucleosides without a contribution from free bases. The amount of adenosine deaminase present in the myocytes is low (estimated by the method of Grever et al. (1983) to be about 2 nmol/min per mg), but sufficient to contribute to conversion of adenosine to inosine in these experiments.

It has been reported that IMP does not accumulate

![Diagram](https://example.com/diagram.png)
in ischemic dog heart tissue (Jennings et al., 1981; Swain et al., 1982; but see Parker et al., 1976) and little IMP is found in globally ischemic rat hearts by our procedures (Table 1). However, IMP increases considerably in anaerobic, glucose-deprived myocytes (Fig. 3; Tables 3 and 4), and this component is increased dramatically when the cells are uncoupled or treated with iodoacetate (Fig. 3). Iodoacetate has been shown to block glycolysis and to increase the release of enzymes from perfused rat heart (Higgins and Bailey, 1983). Since iodoacetate-treated myocytes cannot regenerate ATP by glycolysis, the net production of AMP will be greatly accelerated. A similar situation should prevail in uncoupled cells due to the marked increase in ATPase activity in the presence of CCP. It should be noted that Walker et al. (1982) have reported that rat heart myocytes uncoupled with dinitrophenol degrade ATP almost exclusively to ADP in a 10- to 15-minute incubation. ADP does not show even a transient increase in our studies of uncoupled myocytes (Fig. 4), and the basis for this discrepancy is not certain.

It is not clear why large accumulations of IMP are seen in de-energized myocytes, but not in globally ischemic intact rat heart. It is possible that the myocytes become more completely depleted of high energy phosphate, and since cardiac AMP deaminase activity increases with decreased energy charge (Solano and Coffee, 1978; Ogawara et al., 1980), these conditions would favor IMP production. IMP is present as a normal metabolite in rat heart, since it is an intermediate in the biosynthesis of AN by both de novo and salvage pathways (see Reibel and Rotvett, 1979) and a functional purine nucleotide cycle has been demonstrated in rat heart (Takala et al., 1980).

Reaeration of anaerobic myocytes results in restoration of the energy charge (Hohl et al., 1982) with a small net increase in AN (Table 4). Both a net decrease in adenosine and IMP, and an increase in inosine, accompany the AN increase. Since the anaerobic cells contain only trace amounts of GN prior to reaeration, it seems possible that there is insufficient CTP available to support AN resynthesis from IMP via the purine cycle enzymes (see Fig. 5). The observed increase in AN could result from adenosine kinase activity, however, since the adenylate energy charge is elevated immediately following reaeration. In this case, the removal of IMP could result from 5′-nucleotidase activity, since IMP is a substrate for the cytosolic enzyme in heart (Schutz et al., 1981) and IMP hydrolysis has been shown to increase with increasing energy charge with liver cytosol 5′-nucleotidase (Itoh, 1981). The enzymes involved in these transformations may be affected by P; levels and the free Mg concentration (see Sullivan and Alpers, 1971, for example), so that it is difficult to predict their relative activities under the conditions imposed. Free Mg will be elevated in anaerobic myocytes, since Mg is retained under conditions of severe AN depletion (Table 3). It should also be noted that the absence of erythrocytes and other vascular cells may restrict the metabolic options available to a suspension of myocytes relative to intact tissue. Further work will be necessary to clarify these points.

A preliminary account of a portion of this work has been presented (Geisbuhler et al., 1983).

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