Homogenates of rat heart when fortified with diphosphopyridine nucleotide (NAD) and adenylic acid (AMP) oxidize glucose to CO$_2$ and H$_2$O.$^{1,2}$ This process is associated with a rapid formation of high-energy phosphate bonds.$^{3,4}$ In this preparation occur all of the reactions involved in glycolysis, the tricarboxylic acid cycle, and high-energy phosphate formation and breakdown. Although this system is of great complexity, results obtained with it are highly reproducible and it permits investigation of the roles of different cellular elements and co-enzymes in the metabolism of a substrate.

In earlier study it was observed that a rapid rate of glucose oxidation by the heart homogenate system occurred at a low concentration of ATP and it was concluded that the concentration of ATP present in the myocardium in vivo is far in excess of that necessary for efficient glucose oxidation.$^{5}$ In the present investigation the roles of the adenine nucleotides in the regulation of glucose metabolism in the heart homogenate were studied in more detail. It was found that ATP, when present in a sufficiently high concentration, inhibited glucose utilization and that this inhibition was overcome by magnesium ions. Microsomes were observed to stimulate glucose utilization and to exert this effect at least partly by catalyzing the breakdown of ATP. These studies form the substance of this report.

Methods

Male Wistar rats weighing approximately 200 grams were used. After decapitation, the hearts were rapidly removed and washed in a medium consisting of 0.02 M sodium phosphate (pH 7.2) and 0.123 M KCl. After blotting on filter paper, the hearts were weighed on a torsion balance and homogenized in a glass homogenizer with 1.25 ml of the above medium per 100 mg of tissue. The homogenate was filtered through gauze and used as such or employed for separation of tissue fractions. Incubation was carried out at 37° in air in stoppered Erlenmeyer flasks in a Dubnoff shaker. Each flask contained 0.5 ml homogenate, 0.5 ml 0.15 M KCl, and 0.2 ml of a solution consisting of nicotinamide, NAD, and varying amounts of adenine nucleotides and MgCl$_2$. The exact composition of the reaction mixture varied with the individual experiment. At the end of a period of incubation, 3 ml 0.4 N perchloric acid was added to terminate the reaction. Inorganic phosphate and phosphate hydrolyzed by 1 N HCl at 100° was determined by the method of Fiske and SubbaRow.$^{6}$ Glucose was measured by the glucose oxidase method.$^{7}$ ATP was determined by a specific enzymatic method in which the utilization of ATP in the phosphorylation of 3-phosphoglycerate was coupled to the oxidation of NADH and the reduction of 2,3 diphosphoglycerate in the presence of the appropriate enzymes.$^{7}$ ADP was determined by measuring the reaction with phenazmethyl pyruvate and reducing the pyruvate formed with NADH. After addition of myokinase, AMP was also determined by this method.$^{7}$ NADH was measured in a Beckman spectrophotometer.

In the preparation of tissue fractions the homogenate was first centrifuged at approximately 200 x g for five minutes and the precipitate discarded. This first sediment consisted mainly of red cells, nuclei and partially broken cells. Most of the mitochondria remained in the supernatant fluid which was centrifuged at 12,000 x g for ten minutes. The sediment in the electron microscope consisted...
largely of mitochondria and was designated as the mitochondrial fraction. It was suspended in 0.15 M KCl. The supernatant from this fraction was centrifuged for 30 minutes at 100,000 x g in a Spinco model L ultracentrifuge in order to sediment the microsomes. The supernatant fluid, after removal of the microsomes, was designated as cell sap. All centrifugations were carried out in the cold. In resuspending the mitochondria or microsomes, the sediments were transferred to a glass homogenizer and briefly homogenized in 0.15 M KCl.

Results

UTILIZATION OF GLUCOSE AT LOW CONCENTRATIONS OF ATP

It was observed originally by Wenner et al. that homogenates from kidney, liver, and heart are capable of oxidizing glucose at a considerable rate with NAD as the only co-enzyme added. It is clear from the results of Wenner et al. and from investigations in this laboratory that glucose oxidation may proceed rapidly in a heart homogenate at a low concentration of ATP as long as this compound is constantly regenerated.

The coupling between reactions in the tricarboxylic acid cycle and the utilization of glucose by the heart homogenate is demonstrated by the results reported in table 1.

In these experiments heart homogenates were incubated for various periods of time in the absence of substrate but in the presence of NAD and AMP. Under these conditions there is a rapid formation of ATP associated with oxidation of endogenous substrate. At the end of about 30 minutes, however, the ATP that was formed has almost completely disappeared. As seen from table 1, the preparation at this time is no longer capable of utilizing added glucose. However, if succinate or malate is added at the same time, ATP is regenerated and glucose may be metabolized at a rate equal to that observed in a homogenate system which has not been pre-incubated.

GLUCOSE UTILIZATION AT HIGH CONCENTRATIONS OF ATP

Although glucose utilization may proceed in a homogenate system in which the ATP necessary for the hexokinase reaction is constantly regenerated, glucose is also metabolized when ATP is added initially. Under these conditions, however, it was observed that the rate of glucose utilization was markedly depressed at high concentrations of ATP. These findings are illustrated by the results reported in figure 1. These results were ob-

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of Succinate and Malate on the Utilization of Glucose by Rat Heart Homogenate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Preincubation</th>
<th>Incubation</th>
<th>Cosubstrate</th>
<th>Δ Glucose</th>
<th>ATP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without substrate</td>
<td>+ substrates</td>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0—60</td>
<td>none</td>
<td>4.08</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30—60</td>
<td>succinate (5 mM)</td>
<td>4.01</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30—60</td>
<td>succinate (5 mM)</td>
<td>2.66</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30—75</td>
<td>succinate (5 mM)</td>
<td>0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>30—75</td>
<td>succinate (5 mM)</td>
<td>4.38</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0—30</td>
<td>10—55</td>
<td>none</td>
<td>4.26</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0—10</td>
<td>30—75</td>
<td>succinate (5 mM)</td>
<td>4.08</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0—30</td>
<td>30—75</td>
<td>succinate (5 mM)</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0—30</td>
<td>30—75</td>
<td>succinate (5 mM)</td>
<td>4.33</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>30—75</td>
<td>none</td>
<td>0.12</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30—75</td>
<td>malate (10 mM)</td>
<td>4.40</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30—75</td>
<td>malate (20 mM)</td>
<td>3.53</td>
<td>—</td>
</tr>
</tbody>
</table>

*In all flasks: 0.5 ml homogenate, 0.5 ml 0.15 M KCl, 0.2 ml reaction medium. Total vol = 1.4 ml. 8 μM glucose added. Succinate and malate as indicated. 0.002 M AMP, 0.0006 M NAD, 0.008 M nicotinamide, 0.0002 M MgCl₂. Temp = 37°.

†Initial and final concentrations of ATP are those at the beginning and at the end of the incubation with substrate.
Effect of ATP on the utilization of glucose by rat heart homogenate. Incubation at 37° for 30 minutes. Total vol = 1.4 ml. 0.0006 M NAD, 0.008 M nicotinamide, no MgCl₂, 0.005 M glucose. ATP added in amounts varying from 0.5 to 5.0 μmoles. Abscissa indicates the concentration of ATP after 15 minutes of incubation. Results recorded are from three different experiments, each performed in duplicate.

Effect of ATP on glucose utilization was therefore studied in the presence and absence of various concentrations of added MgCl₂. It was found that, when Mg⁺⁺ was added at a concentration of 0.001 M or higher, glucose utilization was stimulated and the inhibitory action of ATP was completely absent. These experiments are not reported here since the effect of magnesium is well demonstrated in the experiments reported in the next section.

EFFECT OF ADENINE NUCLEOTIDES ON GLUCOSE UTILIZATION

It has been well established that in an energy-coupled system the rate of oxidation of a metabolite is regulated by the availability of the phosphate acceptor, ADP. For this reason it was interesting to determine the concentration of ADP in the reaction system under conditions that made glucose utilization maximal and also in a system inhibited by the addition of a large amount of ATP. Table 2 gives the results of an experiment in which adenine nucleotides were present initially in different concentrations and in which AMP, ADP, and ATP were measured after 15 and 30 minutes of incubation. The experiment was done in the presence and absence of added MgCl₂ and the utilization of glucose was determined.

The results in table 2 show clearly that the addition of ATP initially led to greatly increased concentrations of both ADP and ATP during the period of incubation. In the absence of exogenous magnesium ions glucose utilization was markedly depressed. When ADP, rather than ATP, was added, the concentration of ATP was lower and the disappearance of glucose was less severely inhibited. In the incubation mixtures to which both ATP and ADP were added, very high concentrations of these nucleotides were present during the incubation, and glucose utilization was extremely low. The addition of magnesium ions caused in all cases a stimulation of the metabolism of glucose and made the utilization of this substrate independent of the concentrations of adenine.
The experiment confirms the inhibitory action of ATP and demonstrates convincingly that lack of ADP is not involved in the inhibition of glucose utilization seen at high concentrations of ATP. On the contrary, it is likely that ADP contributes to the inhibition of substrate utilization seen at high concentrations of ADP and ATP.

### EFFECT OF ADENINE NUCLEOTIDES ON OXYGEN UPTAKE

The oxygen uptake of the heart homogenate system was determined in the presence of different amounts of added ADP and ATP. The experimental conditions were identical to those in table 2. The results obtained are recorded in table 3 and show that the concentrations of ATP which inhibited glucose utilization also depressed the oxygen uptake of the preparation. ADP inhibited the oxygen uptake but, as seen when glucose was measured, the metabolic effect was smaller than that of ATP. The addition of magnesium ions overcame the inhibitory action of ATP. It should be noted, however, that the effect of magnesium on oxygen uptake was much smaller than its effect on glucose disappearance (table 2). In separate experiments it has been found that magnesium ions cause an increase in lactate accumulation in a system oxidizing glucose, and it appears that magnesium stimulates principally the conversion of glucose to lactate.

Lactate utilization is not influenced by the concentrations of adenine nucleotides present in these experiments. A homogenate system identical to that used for the experiments in table 3 metabolized lactate rapidly; when ATP was added up to a concentration of 4 μmoles/ml, the rate of substrate utilization did not change significantly.

### EFFECT OF MICROSOMES ON GLUCOSE UTILIZATION

The rate of glucose utilization by the heart homogenate system was determined in the presence of different amounts of added ATP. The experiment was done in the presence and absence of a suspension of microsomes obtained from another heart according to the procedure described in the section on methods. The system contained 0.5 ml homogenate, and the amount of microsomes added...
GLUCOSE UTILIZATION IN HEART HOMOGENATE

TABLE 3

Effect of Nucleotides on the Oxygen Uptake of Rat Heart Homogenate*

<table>
<thead>
<tr>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>MgCl₂ added</th>
<th>Oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles/ml</td>
<td>μmoles/ml</td>
<td>μmoles/ml</td>
<td>μmoles/30 min</td>
<td></td>
</tr>
<tr>
<td>2.04</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.8</td>
</tr>
<tr>
<td>2.04</td>
<td>0</td>
<td>1.43</td>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>2.04</td>
<td>0</td>
<td>2.86</td>
<td>0</td>
<td>3.5</td>
</tr>
<tr>
<td>2.04</td>
<td>1.43</td>
<td>0</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>2.04</td>
<td>2.86</td>
<td>0</td>
<td>0</td>
<td>6.5</td>
</tr>
<tr>
<td>2.04</td>
<td>0</td>
<td>2.86</td>
<td>1.43</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*Experimental conditions identical to those in table 2.

Effect of microsomes on the utilization of glucose by rat heart homogenate. Incubation at 37° for 30 minutes. Total vol = 1.2 ml, 0.0007 M NAD, 0.009 M nicotinamide, 0.006 M glucose. No AMP or MgCl₂ added. The utilization of glucose is plotted as a function of the amount of ATP added initially. Microsomes when added correspond approximately to the amount present in 0.8 ml homogenate.

corresponded approximately to those present in 0.8 ml homogenate. Figure 2 shows that the addition of microsomes caused a large increase in the utilization of glucose at all levels of added ATP. The utilization of glucose was plotted in figure 2 as a function of the amount of ATP added initially, and the effect of microsomes is readily apparent. However, substrate utilization may be expressed also as a function of the concentration of ATP in the reaction medium during the incubation. This has been done in figure 3, where the rate of glucose utilization during the incubation period of 30 minutes was plotted as a function of the concentration of ATP at 15 minutes, the half point of incubation. There is now little effect of microsomes on glucose utilization except possibly at the low concentrations of ATP. It appears that the addition of microsomes to the reaction system decreased the level of ATP and that this was responsible for most of the stimulatory effect of microsomes on glucose utilization.

\[ \text{FIGURE 2} \]

\[ \text{FIGURE 3} \]

Utilization of glucose by rat heart homogenate in the presence and absence of microsomes. The glucose disappearance is plotted as a function of the concentration of ATP at 15 minutes, the half point of the period of incubation. The experiment is the same as that illustrated in figure 2.
EFFECT OF MICROSOMES ON GLUCOSE UTILIZATION AND ATP CONCENTRATION

The effect of microsomes on glucose utilization and acid-labile phosphate formation is illustrated by the experiment reported in figure 4. In this experiment mitochondria suspended in cell sap were incubated with glucose, NAD, and AMP in the presence and absence of added microsomes, and the utilization of glucose and accumulation of acid-labile phosphate were measured. It is seen that microsomes stimulated glucose utilization and depressed net synthesis of acid-labile phosphate during the period of incubation. In the absence of microsomes, i.e., in a system consisting only of mitochondria and the soluble components of the cell, extremely high levels of ATP and ADP were reached and glucose utilization was inhibited.

EFFECT OF MICROSOMES ON GLUCOSE UTILIZATION AT HIGH AND LOW CONCENTRATIONS OF MgCl₂

From the results reported above, it appears that the marked stimulation of glucose utilization by microsomes was caused by the decrease in ATP concentration produced by these tissue components. Since Mg ions have been shown to overcome the inhibitory effect of ATP on glucose utilization, it would be expected that the stimulatory effect of microsomes would be much greater at low concentrations of Mg than at high concentrations of this ion. That this is so can be seen in figure 5. In this experiment glucose utilization of a system consisting of cell sap and mitochondria in the presence and absence of microsomes was measured during a 40-minute incubation period. The rate of glucose utilization was plotted as a function of the ATP concentration at 20 minutes, the half point of the incubation period. The points connected by the lower line represent the results observed at a low concentration of MgCl₂ (0.21 mM). The points connected by the upper line represent the results at a high concentration of MgCl₂ (4.7 mM). In each case the filled symbols indicate the results without microsomes; the open symbols, the
3.0
2.0
1.0
0.5
0.5 1.0 1.5 2.0 2.5
ATP, nMOLES/ML
FIGURE 5
Effect of microsomes on glucose utilization in a cell sap—mitochondrial preparation at high and low concentrations of MgCl₂. Incubation at 37° for 40 minutes. Total vol = 1.2 ml. 0.0007 M NAD, 0.009 M nicotinamide, 0.006 M glucose, 0.0022 M ATP. MgCl₂ 0.00021 M (low) or 0.0047 M (high). Tissue fractions were added in amounts equal to those in the experiment reported in figure 4. Filled symbols correspond to the results without microsomes; open symbols, to the results with microsomes. Abscissa indicates the concentration of ATP at 20 minutes, the half point of incubation.

The experiments reported here confirm our previous conclusions that in cell-free heart preparations glucose may be metabolized at a rapid rate at low concentrations of ATP provided that this nucleotide is rapidly regenerated. When ATP is added initially, or when high concentrations of ATP are built up during the period of incubation, there is a marked inhibition of glucose utilization. This inhibition can be completely abolished by magnesium ions.

The mechanism of the inhibiting action of ATP on glucose utilization is not entirely clear. One likely possibility is that in the presence of high concentrations of ATP and ADP the magnesium ions are complexed with the adenine nucleotides and the concentration of free magnesium ions becomes rate-limiting. Excellent evidence indicates that both ATP and ADP form strong complexes with magnesium. Burton determined free magnesium in mixtures of this metal ion and adenine nucleotides and found that ATP and ADP combined with one magnesium ion. The chelation complexes had dissociation constants of $2.6 \times 10^{-3} \text{ M}$ and $4.5 \times 10^{-4} \text{ M}$ at 25°C and pH 8. This means that the concentration of magnesium in the presence of excess ADP and ATP is extremely low. Studies in which nuclear magnetic resonance was determined have also provided evidence that ATP and ADP form complexes with magnesium. Walaas and Walaas from kinetic studies with partially purified preparations of hexokinase from skeletal muscle concluded that a one-to-one complex of ATP and Mg²⁺ was the actual substrate in the hexokinase reaction.

If there is a lack of free magnesium ions in the presence of high concentrations of ATP, this could lead to a decrease in the rate of the hexokinase reaction which requires magnesium for activity. However, it is possible that a more complicated mechanism is involved. Magnesium ions stimulate the activity of several of the enzymes in glycolysis and increase lactic acid formation in the heart homogenate system. In the absence of added magnesium ions an increase in the concentrations of glycolytic intermediates can therefore be expected. This has been shown in the case of 3-phosphoglyceric acid. This metabolite is present at a considerable concentration in a system to which no magnesium has been added but does not
accumulate in the presence of excess magnesium. It is possible that the concentrations of other intermediates of glycolysis may rise at low concentrations of magnesium and at high levels of ATP and that one or more of these substances may be responsible for the inhibition of the hexokinase reaction. Glucose-6-phosphate has been found to depress glucose utilization in the heart homogenate system, but this substance does not appear to be involved since it is metabolized at a rate which is about eight times that of glucose in a system to which no magnesium ions were added. It was not found to accumulate in the homogenate in the presence of high concentrations of ATP. Further studies are being carried out to determine the possible role of glycolytic intermediates in the regulation of the rate of glucose utilization in the heart homogenate.

The stimulation of glucose utilization by microsomes was observed early in these studies before it was realized that glucose disappearance was inhibited at high concentrations of ADP and ATP. It appears that this metabolic effect of microsomes is caused primarily, if not entirely, by the lowering of the concentrations of ATP resulting from the ATPase activity of these cellular constituents. Whether the endoplasmic reticulum in vivo has a function in the regulation of glucose utilization analogous to the action of microsomes in the in vitro system is unknown.

The inhibition of glucose utilization by ATP is reminiscent of the findings of Passonneau and Lowry, Mansour, and Viñuela et al. that phosphofructokinase is inhibited by high concentrations of ATP. In heart, ATP inhibition of this enzyme can be counteracted by AMP, inorganic phosphate and 3', 5'-cyclic AMP. However, in contrast to the results with glucose utilization, magnesium ions had little effect. It is likely, therefore, that the mechanism of ATP inhibition of phosphofructokinase may be quite different from that reported here.

It is not known whether the effects of ATP and magnesium on glucose metabolism that have been observed in vitro play a role in the regulation of metabolism in the heart in vivo. However, the concentrations of magnesium ions and the adenine nucleotides in the intact heart are such that it is conceivable that phenomena, similar to those described, may occur. The content of magnesium in the rat heart is close to 9 μmoles/g wet weight, and the concentrations of ATP and ADP are about 4-5 and 1 μmoles/g wet weight respectively. Since the concentration of ATP plus ADP is not greatly different from the magnesium concentration, a large proportion of this ion may be chelated in the cell by the adenine nucleotides. It is possible, therefore, that a decrease of the ATP concentration in the heart may cause the release of magnesium ions and stimulation of glucose metabolism.

That such events may occur is also supported by the observations of Regen et al. that anoxia produces a marked increase in glucose phosphorylation and a decrease in the ATP content of the perfused rat heart. The pronounced reduction of cardiac ATP during oxygen lack is particularly well demonstrated in the experiments by Gerlach et al. who showed that the ATP concentration in the rat heart during total anoxia fell to 63% of its original value in 10 minutes. After 20 minutes of anoxia, almost 90% of the original ATP had disappeared. Although the concentration of ADP increased during the first 10 minutes of anoxia, it was less than the initial value at 20 minutes. The sum of the concentrations of ADP and ATP fell continuously during the anoxic period and was only 20% of the initial level at 20 minutes.

Summary

The utilization of glucose by rat heart homogenate may proceed rapidly at low concentrations of ATP when conditions are such that this nucleotide is rapidly regenerated. When high concentrations of ATP are present, glucose utilization is depressed markedly. The inhibition of glucose metabolism is abolished completely by magnesium ions.

Glucose is utilized at a rapid rate by a system consisting of heart mitochondria.
and the soluble components of a heart homogenate. The addition of microsomes to such a system produces a large increase in the rate of glucose disappearance and a decrease in the concentration of ATP. The stimulatory effect of microsomes appears to be mainly a result of an increase in ATPase activity.

A possible role for ATP and magnesium in the regulation of glucose phosphorylation in the intact heart has been discussed.

References
Effects of Adenine Nucleotides and Microsomes on Glucose Utilization in Rat Heart Homogenates

NIELS HAUGAARD, ELLA S. HAUGAARD and ABILIO ANTONIO

Circ Res. 1965;17:135-143
doi: 10.1161/01.RES.17.2.135

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1965 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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/17/2/135