Glucose Oxidation and High Energy Phosphate Production in Heart Homogenates

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The metabolic requirements of a continuously functioning organ such as the heart differ in important respects from those of other structures. It is, therefore, not surprising that many differences in cellular components and metabolism have been observed between the myocardium and other tissues. Thus, in contrast to white skeletal muscle, myocardial cells contain a very high concentration of mitochondria per unit weight. Because of this, the heart is able to oxidize a great variety of substances at a rapid rate and to maintain a high level of adenosinetriphosphate (ATP) by oxidative phosphorylation. It has been demonstrated that the myocardium is capable of keeping the intracellular concentration of ATP constant under conditions during which the heart is markedly stimulated or depressed.

In previous publications, we have described the properties of what may be called a "glucose oxidation system" of rat heart. Homogenates, free of cellular debris, were found to oxidize glucose for many hours at a rate approximating that of the intact heart in vivo. Under optimum conditions, the glucose utilized was completely oxidized to CO₂ and H₂O, and there was little or no accumulation of intermediaries metabolites. When intermediates of the glycolytic and tricarboxylic acid pathways were added to the system, they were found to be oxidized rapidly. It is evident that the heart homogenate is capable of catalyzing efficiently all of the reactions involved in the total oxidation of glucose.

In the presence of a high concentration of diphosphopyridine nucleotide (DPN), glucose oxidation may occur in the absence of other added coenzymes. Under these conditions, we observed that adenylic acid (AMP) was formed slowly from DPN. At low concentrations of DPN, nicotinamide, as an inhibitor of DPNase, and AMP become required cofactors for efficient glucose oxidation.

In the studies described above, it was noted that, despite the well-known role of ATP in the hexokinase reaction, the capacity of this coenzyme to stimulate glucose oxidation was no greater than AMP. To reach a better understanding of the role of AMP and ATP in glucose oxidation, we carried out experiments in which the esterification of inorganic phosphate was measured. It was found that when glucose oxidation proceeded in the absence of AMP, there was only a small formation of acid-labile phosphate esters (ADP and ATP). However, when AMP was added to the reaction system, there was an immediate synthesis of ADP and ATP. High levels of these compounds were reached in a few minutes and could be maintained for an hour or more. It is therefore possible under these conditions to study glucose oxidation and the formation and storage of high energy phosphate compounds in the presence of all the major components of the cell. The results of such studies are presented here.

Methods

Hearts were removed from decapitated rats and washed in cold salt solution of the composition described below. After blotting on filter paper and weighing on a torsion balance, the hearts were minced with scissors and homogenized in an iced glass homogenizer with a relatively loose pestle.
The homogenization medium had the following composition: 0.123 M KCl, 0.020 M sodium phosphate (pH 7.2); 1.0 or usually 1.5 nil. medium was added per 100 mg. tissue. To stabilize the preparation, ethylenediamine tetra-acetic acid (EDTA), disodium salt, was added to give a final concentration of $5 \times 10^{-4}$ M. The homogenate was then filtered through gauze. In some experiments, the sediment obtained after centrifugation at 200 X G for five minutes was discarded. On examination under the phase microscope, this fraction was found to consist of cells in various stages of disruption, myofibrillar material, nuclei, and red cells. A sample of the centrifuged homogenate was examined under the electron microscope by Dr. Richard Davis of this department. The homogenate was found to be almost free of myofibrils and cellular fragments and to contain a large concentration of mitochondria. A major proportion of these were relatively undamaged, and others were in various stages of disruption. The homogenates thus prepared were added to reaction mixtures that varied considerably in the different experiments. However, in a typical experiment, the system consisted of the following: 0.5 ml. homogenate (33 or 50 mg. tissue), 0.5 ml. 0.15 M KCl, 0.02 ml. 0.1 M MgCl₂, 0.05 ml. 5 per cent glucose, and AMP, DPN, or other coenzymes as indicated. Oxygen uptake was determined in Warburg vessels with alkali in the side compartment. In the experiments on phosphorylation, the reaction mixtures were incubated in Erlenmeyer flasks in a Dubnoff shaker. The reaction was stopped by the addition of 5 ml. 0.4 M perchloric acid. Inorganic phosphate and phosphate hydrolyzed by 1 N HC1 at 100 C. for 10 minutes were determined by the method of Fiske and Subbarow.8

Results

**Utilization of Inorganic Phosphate**

In the experiments on glucose oxidation by Wenner et al.10 and in our earlier studies,5,6 DPN was present in a large concentration and was the only coenzyme added. Under these conditions, there was little change in the inorganic phosphate concentration, although, after an initial lag period, there was a rapid oxidation of glucose. When AMP was added in addition to DPN, a high linear rate of glucose oxidation was established immediately. AMP was maintained when glucose was oxidized, but when glucose was absent from the reaction mixture, there was a rapid breakdown of AMP to adenosine and inorganic phosphate. The acid-labile phosphate was not measured in these experiments. In later experiments reported here, it was found that in the presence of AMP, large amounts of acid-labile phosphate appeared within the first few minutes of incubation, indicating that ADP and ATP were formed. The initial disappearance of inorganic phosphate was found to be greater the larger the amount of AMP added to the reaction medium, and was equivalent in amount to the acid-labile phosphate formed. To identify the acid-labile phosphate compounds, we carried out the experiments described in the next section.

**Demonstration of ADP and ATP Formation**

A heart homogenate was incubated for five minutes in the presence of C⁴-AMP, *glucose, DPN, and nicotinamide. At the end of the incubation, the reaction was stopped by the addition of perchloric acid. In an initial control, the incubation was omitted. The Ba-insoluble compounds were precipitated according to the procedure described by LePage.11 Measurements of radioactivity were made of the original perchloric acid filtrate and of the Ba-precipitate after it was dissolved in acid. The Ba-insoluble nucleotides were adsorbed on charcoal, eluted with pyridine-alcohol, and an aliquot of the eluate was chromatographed on paper at 4 C. together with carrier amounts of ADP, ATP, and DPN. The ascending technique was used with the solvent system isobutyric acid, ammonia, water (66:1:33), and the spots corresponding to ADP, ATP, and DPN were eluted and counted. The results of the experiment are given in table 1.

*C⁴-AMP was obtained from Schwarz BioResearch, Inc.
the AMP moiety. On chromatography of the compounds eluted from the charcoal, the radioactivity was present in the ADP and ATP spots, except for a barely significant amount at the site of the DPN. When the amount of radioactivity recovered was corrected for the loss on charcoal adsorption and chromatography, the AMP present (as ADP and ATP) corresponded approximately to that expected if the phosphate utilized had combined with AMP.

In another experiment in which nonlabeled AMP was used, it was found that all of the acid-labile phosphate was present in the Ba-insoluble fraction. This excluded glucose-1-phosphate which is not precipitated with barium in the absence of alcohol.11

We have concluded from these experiments that the formation of acid-labile phosphate observed is a measure of the production of ADP and ATP from AMP.

CHANGES IN PHOSPHATE

Figure 1 illustrates the changes in inorganic phosphate occurring in the heart homogenate system in the presence and absence of glucose. In the complete system, a disappearance of phosphate occurs during the first few minutes of incubation. The phosphate concentration is then maintained at a constant level. Without added glucose there is a small disappearance of phosphate which is promptly reversed and followed by a steady increase in phosphate concentration. The increase in inorganic phosphate is caused by the action of 5'-nucleotidase, an enzyme which, in contrast to skeletal muscle, is present in the heart in a very high concentration.7 When glucose was added after 20 minutes to the system incubated without substrate, the appearance of phosphate ceased, and inorganic phosphate was again utilized.

In the same experiment, we also determined "acid-labile" phosphate, i.e., the difference between the concentration of inorganic phosphate and the phosphate concentration after hydrolysis for 10 minutes in 1 N HCl at 100 C. The results of these experiments are presented in figure 2.

A high concentration of acid-labile phosphate (ADP and ATP) is rapidly reached

TABLE 1

Formation of ADP and ATP from C14-AMP in Rat Heart Homogenate During Glucose Oxidation

<table>
<thead>
<tr>
<th>Formation of acid-labile phosphate (µmoles)</th>
<th>Appearance of Ba-insoluble adenosine nucleotides (µmoles)</th>
<th>Ba-insoluble nucleotides determined by paper chromatography</th>
<th>Calculated acid-labile phosphate in ADP and ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>+0.30</td>
<td>+0.07</td>
<td>+2.39</td>
<td>+5.43</td>
</tr>
</tbody>
</table>

*Incubation: five minutes at 37 C.

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and maintained during glucose oxidation. In the absence of added glucose, the ADP and ATP formed initially are soon broken down but can be reformed by the addition of glucose to the reaction system. DPN is a necessary cofactor; in its absence no phosphorylation of AMP occurs. Dinitrophenol causes complete inhibition of the reaction at a concentration of $5 \times 10^{-3}$ M. These observations, together with the finding that the glucose utilized by the system is completely oxidized to CO$_2$ and H$_2$O,$^5$-$^7$ support the conclusion that the high energy phosphate compounds formed in the system are produced not only by the reactions of glycolysis but by oxidative phosphorylation as well.

**CAPACITY TO FORM ATP**

The amount of acid-labile phosphate formed was found to increase as the concentration of added AMP was increased. Such an experiment is illustrated in figure 3. Utilization of inorganic phosphate was measured during incubation for five minutes of a glucose-oxidation system containing a variable concentration of AMP.

It is seen that the extent of the reaction is proportional to the initial concentration of AMP. The slope of the curve at zero AMP concentration is close to 2, indicating that at low concentrations of AMP this compound may be completely converted to ATP.

**RELATION BETWEEN GLUCOSE OXIDATION AND PHOSPHORYLATION**

One of the most interesting problems raised by the observations reported here is the relation between the concentration of ATP in the reaction system and the ability of the heart homogenate to oxidize glucose. This was studied in experiments reported in figure 4.

Oxygen uptake of the glucose-oxidation system was studied, and parallel experiments were done in which changes in acid-labile phosphate were determined. As found previously, a rapid oxidation of glucose may occur in the absence of added AMP. As seen
in figure 4, under this condition there was a significant, but very small, increase in acid-labile phosphate (ADP and ATP), while in the presence of AMP there was a large accumulation of ADP and ATP. It can be concluded from these experiments that a rapid rate of glucose oxidation may occur in the presence of a low concentration of ATP. However, when AMP is added in excess, the system is capable of synthesizing and storing large amounts of ADP and ATP.

ACID-STABLE PHOSPHATE ESTER FORMATION

Experiments in which acid-stable ester formation was measured supported the finding that the initial rapid disappearance of inorganic phosphate in the system corresponded to the formation of ADP and ATP and that glucose was oxidized with little accumulation of phosphorylated intermediates. On continued incubation, however, particularly under conditions at which a high concentration of ATP was reached, there was a slow formation of acid-stable phosphate intermediates. This is a problem for future experimentation.

Discussion

Our purpose in these experiments was to investigate the factors which regulate the rate of glucose oxidation and energy production in the heart. There is good reason to believe that the homogenate system, which contains the major components of the cell, oxidizes metabolites in a manner and to an extent approximating that found in the intact cell.

The qO₂ of our homogenates, for example, under optimal conditions of glucose oxidation, is approximately 60. This figure is similar to the figure of 54.0 given in the literature for the beating dog heart.2

Our studies have been concerned mainly with the oxidation of glucose. However, a number of intermediates of carbohydrate metabolism, such as phosphorylated hexoses, lactic acid, and members of the tricarboxylic acid cycle, are also oxidized rapidly.5,6 The oxidation of fatty acids3 and an amino acid such as aspartic acid12 may also be studied in properly fortified heart homogenates.

The experiments demonstrate that the oxidation of glucose is associated with a very rapid generation of high energy phosphate in the form of ADP and ATP. It can be calculated from the data in figure 2 that the rate of high energy phosphate (P − P) formation is about 2,000 μmoles/Gm./hr.; even higher rates have been observed. The oxygen uptake of a glucose oxidation system similar to that studied in the experiment reported in figure 2 is close to 500 μmoles/Gm./hr. When this figure is used, the calculated P/O ratio is about two. This indicates that a reasonably efficient coupling between oxidation and phosphorylation exists in these experiments.

During further incubation of the homogenate system, high levels of ADP and ATP are maintained as long as respiration proceeds. If sufficient AMP is added, these concentrations may reach values close to those found in the intact cell.3 However, the homogenate contains a high concentration of ATPase. In homogenate systems similar to the one in which phosphorylation was studied, but in the absence of DN and glucose, the rate of breakdown of ATP was found to be of the same order of magnitude as the rate of high energy phosphate formation. The levels of ADP and ATP maintained in the system are, therefore, dependent on the relative rates of these two processes. As we have mentioned,
heart homogenates, in contrast to skeletal muscle preparations, contain a high concentration of 5'-nucleotidase. This enzyme, which causes the splitting of AMP to adenosine and phosphate, is associated with microsomes and is responsible for the rapid formation of inorganic phosphate in the absence of glucose oxidation.

The efficiency of AMP as a phosphate acceptor in the system is remarkable. It is usually considered that ADP, rather than AMP, is the primary phosphate acceptor in oxidative phosphorylation. No lag period of phosphorylation of AMP could be observed in these experiments, even when the temperature of the incubation was lowered to 20°C. This indicates either that AMP is directly phosphorylated or that it is used with great rapidity to provide a source of ADP through the myokinase reaction: ATP + AMP = 2 ADP. The latter mechanism cannot be excluded since the homogenate contains small amounts of endogenous ATP. ADP formation from AMP may conceivably take place extremely rapidly on the surface or inside mitochondria where the concentration of ATP may be high. Whatever the mechanism, these experiments demonstrate the marked efficiency of AMP as a phosphate acceptor in heart tissue. Ochoa also found that AMP functions as a phosphate acceptor in experiments with heart extracts oxidizing pyruvate.

Some important conclusions about the initial step in glucose oxidation may be drawn from these experiments. Hexokinase has been demonstrated to be present in heart tissue, and the hexokinase reaction is probably the first reaction in the oxidation of glucose in this system. If this is so, our experiments demonstrate that this reaction can proceed at a high rate in the presence of a low concentration of ATP. This may mean that the phosphorylation of glucose takes place, not in the soluble part of the system, but on the mitochondria where newly formed ATP, or high energy phosphate in a form different from ATP, is directly available to the glucose molecule at the appropriate enzymatic surface. According to this view, the high level of ATP (0.007 M) present in the cardiac cell is not necessary for rapid glucose oxidation.

The initial phosphorylation appears to be a limiting factor in the total oxidation of glucose, since there is little or no accumulation of intermediates in a rapidly respiring system. This is in agreement with the observations of Park and coworkers who found that in a perfused rat heart, in the presence of insulin, there is a fast transfer of glucose across the cell membrane and an accumulation of free glucose inside the cell.

It is of interest that the concentration of mitochondria is a limiting factor in determining the rate of glucose oxidation in the homogenate system. In experiments in which the mitochondria were separated and added back to the soluble part of the system, the rate of glucose oxidation was strictly proportional to the concentration of mitochondria. In contrast, at a constant concentration of mitochondria, the supernatant fluid (microsomes + soluble enzymes) can be reduced by 20 per cent with very little effect on the rate of oxidation. These findings give support to the view that the initial phosphorylation of glucose takes place on or inside the mitochondrion.

Our studies show that the factors influencing the complete oxidation of glucose and the phosphorylation coupled with this metabolic sequence may be studied under circumstances that approximate the conditions in the intact cell. The reactions occur with remarkable rapidity and are influenced by the concentration of the coenzymes AMP and DPN, as well as by the concentration of inorganic phosphate and cations. In the present study, we have described the essential characteristics of the system and pointed out certain conclusions which may be drawn about myocardial glucose oxidation and phosphorylation. The homogenate system should be of great usefulness in future studies of normal and abnormal cardiac metabolism.

Summary
Experimental conditions have been established for the study of glucose oxidation and
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high energy phosphate formation in cell-free rat heart homogenates. On addition of AMP to an otherwise complete system, an immediate formation of ADP and ATP occurs. Glucose oxidation is rapid, and high levels of ADP and ATP are maintained for long periods of time. In the absence of added AMP, rapid glucose oxidation also occurs despite low levels of ATP. It can be concluded that the high concentration of ATP, which the heart tissue is capable of accumulating, is far in excess of that necessary for efficient glucose oxidation.

References
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