Intracellular Compartmentation of Glycolytic Phosphate Esters*

N. KALANT and R. BEITNER‡

From the Lady Davis Institute for Medical Research of the Jewish General Hospital and the Department of Experimental Medicine, McGill University, Montreal, Canada

SUMMARY

Rat diaphragm was incubated with [14C]-glucose or [14C]-pyruvate; a number of the phosphorylated glycolytic intermediates were then isolated and their specific activities determined. Following incubation with either substrate, the specific activities of fructose 6-phosphate, fructose 1,6-diphosphate, and phosphoeno pyruvate were very close to that of the substrate. The specific activities of glucose 6-phosphate and phosphoglyceric acids were much lower than those of the substrates and of their immediate precursors; their relative specific activities were different for the two substrates. It is concluded that there are multiple intracellular pools of glucose 6-phosphate and phosphoglyceric acids and that the pools involved in glycolysis are distinct from those involved in gluconeogenesis. Insulin had no effect on the pattern of specific activities.

It has been shown in studies with liver homogenates (1) and hemidiaphragm (2) that glucose-6-P added to the incubation medium stimulates, rather than inhibits incorporation of glucose into glycogen. This was interpreted as evidence that glucose-6-P is not an obligatory intermediate in the synthesis of glycogen. It was later shown (3) that glucose-6-P also stimulated incorporation of glucose into CO2; the conclusion here also was that glucose-6-P is not an obligatory intermediate in glucose oxidation and a metabolic pathway which excluded glucose-6-P was proposed to explain the results.

Shaw and Stadie (4) had previously obtained evidence that in the rat diaphragm there are two discrete Embden-Meyerhof pathways, one which they believed to be intracellular, the other to be on the plasma membrane. They thus postulated the presence of two pools of each of the components of the glycolytic pathway. In attempting to confirm these conclusions, Sims and Landau (5) and Antony et al. (6), working with the diaphragm, obtained results which could be interpreted either as evidence for a glycogenetic pathway which excludes glucose-6-P, or as evidence of the presence of two pools of glucose-6-P within the cell. Similar tentative conclusions of compartmentation of glucose-6-P were reached for skeletal muscle (7) and for liver (8, 9). Recently, Threlfall and Heath (10) obtained more conclusive evidence that in the liver, both glucose-6-P and triose phosphates are compartmented into glycolytic and gluconeogenic pools. The present work was undertaken to determine whether similar compartmentation occurs in the diaphragm.

MATERIALS AND METHODS

Uniformly labeled [14C]-glucose, 261 mCi per mmole, and sodium-[1-14C]-pyruvate, 27.2 mCi per mmole, were obtained from the Amersham/Searle Corporation. The [14C]-glucose, as supplied, was contaminated with a variety of phosphorylated glycolytic intermediates; it was therefore purified before each experiment by preparative thin layer chromatography on cellulose, as described below.

Other materials, the preparation and incubation of rat hemidiaphragms, the extraction and precipitation of phosphorylated glycolytic intermediates, and analytical measurements were as described previously (11).

Separation of Glycolytic Intermediates—Following separation of the barium- and alcohol-precipitable phosphate esters, 3-phosphoglyceraldehyde and dihydroxyacetone phosphate were hydrolyzed by incubation in 1 N KOH for 20 min at room temperature (12). The solution was then neutralized with HCl and subjected to thin layer chromatography for separation of the remaining phosphorylated compounds. The chromatographic technique of Grassetti, Murray, and Wellings was employed (13) with two modifications: the "sandwich" system was not used, and long plates (40 cm), allowed to develop to a height of 38 to 40 cm, were used in order to obtain good separation of fructose-6-P and fructose-di-P. Standards applied at the sides of each plate were visualized with the reagent of Hanes and Isherwood (14) for guidance in the elution of the sample; appropriate areas were eluted in 1.3 ml of water. 2-P-glyceric acid and 3-P-glyceric acid were eluted together. Glucose-6-P and glucose-1-P, which were not well separated, were subjected to a second chromatographic system as follows. They were eluted together and the glucose-1-P was hydrolyzed for 7 min in 1 N HCl at 100° (12). The sample was then freeze-dried, dissolved in 50 μl of H2O and separated on thin layer chromatography plates prepared on microscope slides coated with DEAE-cellulose (15) (7.5 g of DEAE-cellulose, 7.5 g of cellulose, Macherey-Nagel type MN 300, and 120 ml of water were mixed in a blender, applied to the plates, and allowed to dry overnight at room temperature). The sample was spotted 0.7 cm from the bottom and the solvent was allowed to rise to a height of 6.5 cm. The
TABLE I

Percentage of recovery of glycolytic phosphate esters added to diaphragm homogenate

The results represent the mean ± the standard error in seven experiments.

<table>
<thead>
<tr>
<th>Glycolytic phosphate esters</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>Glucose-6-P</td>
<td>93 ± 1</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>Fructose-1,6-di-P</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>3-P-glyceric acid</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>2-P-glyceric acid</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>93 ± 3</td>
</tr>
</tbody>
</table>

TABLE II

Specific activities of phosphorylated glycolytic intermediates after incubation with glucose

Values are mean ± standard error of four experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative specific activity (glucose in medium = 1)</th>
<th>p value for insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>0.129 ± 0.009</td>
<td>&gt;0.4</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>0.144 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-di-P</td>
<td>0.997 ± 0.030</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>3-P-glyceric acid</td>
<td>0.946 ± 0.038</td>
<td></td>
</tr>
<tr>
<td>2-P-glyceric acid</td>
<td>0.982 ± 0.027</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>3-P-glyceric acid plus 2-P-glyceric acid</td>
<td>0.917 ± 0.034</td>
<td></td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>0.936 ± 0.030</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

In each experiment, hemidiaphragms from 14 rats were incubated for 2 hours in Krebs-Ringer-bicarbonate buffer containing glucose at a concentration of 5.56 mM and [14C]-glucose to give a final specific activity of 325 dpm per nmole or with carrier pyruvate at a concentration of 25 mM and [14C]-pyruvate to give a specific activity of 500 dpm per nmole. Insulin was added to one hemidiaphragm of each pair to give a final concentration of 1 × 10^-6 units per ml. After the incubation, the washed hemidiaphragms were frozen and pooled. Specific activities are determined for glucose or pyruvate in the medium and for the phosphorylated compounds as previously discussed. The results (Tables II and III) following incubation with either glucose or pyruvate show specific activities of fructose phosphates and P-enolpyruvate almost equal to that of the glucose in the medium indicating that equilibrium had virtually been reached. From precursor-product relationships in the presence of a continuous source of label we would expect the specific activity of each compound to be equal to, or lower, than that of its product. Yet the specific activities of glucose-6-P and P-glyceric acids were much lower than those of their products. These results strongly suggest that each of these two compounds
exists in multiple intracellular pools. One pool must have been labeled to the same extent as the respective product (fructose-6-P and P-enolpyruvate). The procedure used for estimation of specific activity is based on the total amount of the compound in the tissue and therefore gives an average value for all hypothetical pools. The low specific activities observed must therefore have been due to unlabeled or minimally-labeled pools of glucose-6-P and P-glyceric acid. This is in agreement with the concept of multiple pools of glucose-6-P previously suggested for the liver (8-10).

Shaw and Stadie (4) have postulated the existence of two Embden-Meyerhof pathways in rat diaphragm differing in cytological location, one pathway being intracellular and the other located on the cell surface. They derived their conclusion from experiments in which they compared the metabolic fate of [14C]-glucose and the response to insulin of rat diaphragms incubated in two different buffers: phosphate-0.9% NaCl solution or bicarbonate-phosphate-saline medium. These authors did not purify the [14C]-glucose; in view of our finding that it may be contaminated by phosphorylated intermediates which can seriously interfere with the determination of specific activity, it might be of value to repeat their work with more highly purified preparations.

Dully, Bocek, and Beatty (7) and Sims and Landau (5) suggested the existence of at least two pools of glucose-6-P in voluntary skeletal muscle and diaphragmatic muscle. Their results, however, cannot exclude an alternative interpretation, the existence of a pathway from glucose to glycogen without glucose-6-P as an intermediate. In a recent report, Antony et al. (6) incubated rat diaphragms with a mixture of [6-14C]-glucose and [1-14C]-pyruvate and compared the distribution of 14C from the substrates in the various carbons of glycogen and glucose-6-P; they found a greater incorporation of glucose carbon, relative to pyruvate carbon, into glycogen than into glucose-6-P. This result suggests again the existence in muscle of more than one pool of glucose-6-P or of a pathway from glucose to glycogen without glucose-6-P as an intermediate. Our results showing that the specific activity of glucose-6-P was much lower than those of fructose-6-P and fructose-di-P indicate the existence of two or more glucose-6-P pools in the diaphragm or the existence of a pathway from glucose to fructose-6-P without glucose-6-P as an intermediate. Metzger et al. (3) have proposed such a pathway; however, the 2-hour incubation period would obviously have been adequate to allow equilibration of [4C]-glucose-6-P with [14C]-fructose-6-P regardless of the pathway by which the latter was produced. Since isotopic equilibration did not occur, it is concluded that fructose-6-P is not in metabolic equilibrium with the total pool of glucose-6-P and that there are at least two pools of glucose-6-P.

The present results also indicate the existence of more than one pool of P-glyceroic acids in the diaphragm muscle. Threlfall and Heath (10) have found that in the liver of rats injected with uniformly labeled [14C]-fructose, the specific activities of glucose-6-P and glycerol-1-P were lower than those of glucose and UDP-glucose; from a kinetic analysis of the results, they concluded that the glucose-6-P and the triose phosphate pools are compartmented into gluconeogenic and glycolytic components. To determine whether similar compartmentation exists in muscle, the experiments with labeled pyruvate were carried out. The specific activities of the glucose-6-P and P-glyceroic acids were again lower than those of the other intermediates, whose specific activities were very close to that of the pyruvate in the medium. In comparing the glucose and pyruvate experiments (Table IV), it is seen that the ratio of specific activities of fructose-6-P, fructose-di-P, and P-enolpyruvate to that of the substrate in the medium during glycolysis were essentially the same as those during gluconeogenesis. The fraction of the total pool of each compound which is involved in glycolysis or gluconeogenesis is therefore related to the relative specific activity of the total pool. Since for glucose-6-P, the pool fraction involved in glycolysis is not the same as that involved in gluconeogenesis, it must be concluded that these are separate subpools. Furthermore, since the sum of the two subpools is only 0.38 of the total, it may be concluded that there is at least one more pool of this compound. Similar conclusions apply to P-glyceroic acid.

Previous experiments carried out under the same conditions as the present ones showed that insulin increased the concentrations of fructose-1,6-di-P, P-glyceroic acids, and P-enolpyruvate. However, in the current experiments, insulin had no effect on the specific activities of these compounds. It must therefore be concluded, in the case of P-glyceroic acids, that insulin increased the pool size not only of that pool directly involved in glycolysis, but also of the other pools of this compound.

The interaction of glycolysis and gluconeogenesis in diaphragm can be represented by the scheme shown in Fig. 1. Pools of glucose-6-P and P-glyceroic acid involved in glycolysis are separated from those involved in gluconeogenesis. The triose phosphates were not studied in the present experiments, but experiments on liver (1) indicate that they too are compartmented. Whether the various pools are localized at specific sites within the cell remains to be determined. The presence of compartmentation complicates considerably the interpretation of certain types of metabolic investigations.

REFERENCES
