Hepatic hexose transport and the effect of N₂-induced anoxia and KCN on this process

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(Received 6 September 1984)

Hepatic hexose transport was characterized using 3-O-methyl-D-glucose, which is not metabolized by the liver. The kinetic parameters determined in the starved state were taken as basal values for the transport system which showed saturation kinetics with high Vmax and Km values of 161 nmol/mg dry wt./min and 39 mM respectively. In the fed state, the Vmax was found to be increased nearly two-fold; this may be due to a phenomenon known as trans-stimulation. The effects of N₂-induced anoxia and of KCN were investigated. In the fasted state, anoxia caused the transport characteristics Vmax and Km to decrease nearly two-fold whereas KCN had the opposite effect as the Vmax and Km were increased by three- and two-fold respectively. In the fed state, anoxia and KCN caused a marked decrease in the transport characteristics.

Liver perfusion studies in rats (1) and in dogs (2) have shown that D-glucose rapidly equilibrates between the liver and blood and that a stereospecific hexose-transport process exists in this organ. However, perfusion systems present technical difficulties in the study of transport phenomena across plasma membranes and, hence, the use of hepatocytes now predominates in hepatic hexose-transport studies (3,4). Baur and Heldt (3) and Craik and Elliott (4) have shown the existence of a passive facilitated diffusion mechanism for the transport of hexoses across the plasma membrane of isolated parenchymal cells. In this investigation, 3-O-methyl-D-glucose (which is not metabolized by hepatocytes) has been used to study the transport process in cells in two different metabolic states - depleted of glycogen and replete with glycogen.

Although the rate of transport of hexoses into liver cells largely exceeds its metabolic conversion (3), in both skeletal and cardiac muscle the transport process is the rate-limiting step in hexose metabolism and is stimulated by anoxia or complete inhibition of

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oxidative metabolism (6,7). Thus the effect of partial and complete inhibition of oxidative metabolism, using \( \text{N}_2 \)-induced anoxia and KCN respectively, was investigated in hepatocytes prepared from fasted and fed rats to see if a similar event occurred.

**Materials and Methods**

Male Porton-Wistar rats (150-200 g) were used: some were allowed free access to food and others were starved for 24 h. 3-O-methyl-D-glucose and inulin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Collagenase (Grade II) was from C.F. Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K. Dow Corning Silicone fluid (DC550) of density 1.07 g/ml was obtained from Hopkin & Williams, Chadwell Heath, Essex, U.K. [U-\( ^{14} \text{C} \)]-3-O-methyl-D-glucose (sp. radioactivity 74.2 mCi/mMol), \( ^{3} \text{H}_2\text{O} \) (sp. radioactivity 10 Ci/ml \( \text{H}_2\text{O} \)), and \( ^{3} \text{H} \)-inulin (sp. radioactivity 1.09 Ci/mMol) were obtained from Amersham International p.l.c., Bucks., U.K. All other chemicals were of the purest grade available from standard suppliers.

**Preparation of isolated hepatocytes**

Isolated hepatocytes were prepared by perfusion of the liver with collagenase as described by Krebs et al. (8), with minor modifications (9). After collagenase treatment hepatocytes were suspended in Krebs-Ringer bicarbonate (10) pH 7.4 containing 2% dialysed bovine serum albumin (Fraction V). Viability of hepatocyte preparations was assessed in several ways (9). First, Trypan blue was excluded by 90-95% of cells. In addition, rates of gluconeogenesis from various precursors were found to be similar to those reported in other laboratories. Preceding 3-O-methyl-D-glucose uptake studies, hepatocytes were incubated for 20 min in a shaking water bath at 37°C and gassed with 95% \( \text{O}_2 \) + 5% \( \text{CO}_2 \) to allow cells to stabilize and to achieve a constant metabolic state. Liver cells were then incubated for 5 min at 22°C prior to addition of labelled 3-O-methyl-D-glucose solution (gassing with 95% \( \text{O}_2 \) + 5% \( \text{CO}_2 \) throughout). In the case of \( \text{N}_2 \)-induced anoxia, cells were gassed with 95% \( \text{N}_2 \) + 5% \( \text{CO}_2 \) for 5 min both after the initial 20-min incubation period and during transport studies. In the case of respiratory inhibition, KCN was added to the liver-cell suspension to give a final concentration of 5 mM and incubated for 1 min before addition of labelled substrate solution.

**Transport measurements**

The technique used for measuring uptake of 3-O-methyl-D-glucose was that of an oil centrifugal filtration method as described by Baur and Heldt (3). Transport was initiated by rapid addition of 3-O-methyl-D-glucose containing radioactive tracer (0.1 \( \mu \text{Ci/ml} \)) to 1.5 ml (17 mg dry wt./ml) of cell suspension (kept at 22°C) so as to give hexose concentrations ranging from 5 mM to 30 mM. For each substrate concentration, duplicate uptake measurements were taken. Samples (150 \( \mu \)l) were taken at the appropriate time and centrifuged at 1000 g through (70 \( \mu \)l) silicone fluid (density 1.07 g/ml) into
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(20 μl) 1.6-mM HClO₄ (density 1.47 g/ml) using a Beckman Microfuge B. The amount of 3-O-methyl-D-glucose taken up into the filtered cells was determined from the radioactivity measurements in the sediment fraction. Initial rates of uptake of 3-O-methyl-D-glucose were determined for each substrate concentration by taking tangents to the progress curves (Figs. 1 and 2).

In parallel experiments the uptake of [³H]inulin and ³H₂O was measured. (Cells were exposed to either [³H]inulin or ³H₂O for 3 min prior to centrifugation through oil.) This allowed the determination of sugar uptake into the inulin-impermeable ³H₂O space (this was found to be 1.3 μl/mg dry wt. for hepatocytes prepared from both fed and fasted animals).

Statistical treatment of experimental data

Results were initially subjected to linear regressional analysis on a Lineweaver-Burk plot (Type I). However, evidence published by Wilkinson (11) indicates that such a transformation of the Michaelis-Menten equation may be statistically objectionable since severe weighting may occur on data points at low substrate concentrations. Therefore, Vₘₐₓ and Kₘ were also determined from a direct linear plot (Type II) using a Commodore Pet 2001 series computer program based on work by Eisenthal and Cornish-Bowden (12). Note that no S.E.M. could be obtained using this method.

Determination of glycogen and glucose content

Glycogen content of hepatocytes was determined using amylglucosidase as described by Lee and Whelan (13). Glucose concentrations were determined using the aminophenazone method (14).

Results and Discussion

In hepatocytes prepared from starved animals the levels of glycogen and glucose are low (1.35 μmol of glycogen-glucose/g wet wt. of cells and 4.75-mM glucose respectively). The kinetic parameters obtained in this state using 3-O-methyl-D-glucose were considered to be the basal characteristics of the transport system and are shown in Fig. 1. The kinetic parameters found under differing metabolic conditions were then determined and compared to those found in the fasted state. Fig. 2 shows the uptake of 3-O-methyl-D-glucose into hepatocytes prepared from fed animals where the liver is full of glycogen (347.5 μmol of glucose-glycogen/g wet wt. of cells) and the intracellular glucose concentration is high (9.25 mM). Figs. 1 and 2 show that the initial rates of uptake are rapid and that the system begins to equilibrate after about 15 s. However, in cells prepared from fed animals (Fig. 2 and Table 1) the initial rates of uptake are higher than those found in cells prepared from fasted animals at the same substrate concentration. Note that approx. 5% of the total uptake is attributable to a non-facilitated diffusion process (3).

The kinetic parameters obtained from Type-I analysis of untreated cell data (Table 2) show that the Vₘₐₓ and Kₘ are respectively 1.8- and 1.4-fold higher in the fed state compared to the fasted state. However, when Type-II analysis was used, only the Vₘₐₓ was higher in
Fig. 1. Time courses of uptake of 3-O-methyl-D-glucose in hepatocytes prepared from 24-h-starved rats. Hepatocytes were prepared from 24-h-starved animals and pre-incubated at 37°C. Measurement of uptake was then carried out at 22°C, gassing with 95% O₂ + 5% CO₂ using 3-O-methyl-D-glucose concentrations of 5 mM (O), 10 mM (●), and 15 mM (■). Results are means, and bars indicate the S.E.M. Data were obtained from 4 experiments, and assays were performed in duplicate.

the fed state and no difference in the Kₘ values was observed. Therefore, probably only an increase in Vₘₐₓ occurs as a consequence of feeding the animals. This increase in hexose uptake in the fed state may be due to the phenomenon known as 'trans-stimulation' which is known to occur in human erythrocytes when the cytosolic hexose concentration is high (15,16). This process occurs as a result of intracellular hexose stimulating the transport system in such a way as to increase the rate of entry of the hexose being measured. This phenomenon may also occur in the opposite direction whereby extracellular hexose stimulates the rate of hexose efflux from the cytosol. Trans-stimulation has also been observed in transport studies.

Fig. 2. Time courses of uptake of 3-O-methyl-D-glucose in hepatocytes prepared from fed rats. Hepatocytes were prepared from fed animals and pre-incubated at 37°C. Measurements of uptake using 5-mM (O), 10-mM (●), and 15-mM (■) 3-O-methyl-D-glucose were then performed at 22°. Cells were continuously gassed with 95% O₂ + 5% CO₂. Results are the means obtained from 5 experiments (performed in duplicate) and the bars represent the S.E.M.
Table 1. 3-O-methyl-D-glucose in cells prepared from 24-h-starved and fed rats

Hepatocytes were prepared from 24-h-starved and normally fed animals and pre-incubated in a shaking water bath at 37°C. Uptake measurements were carried out at 22°C, gassing with 95% O₂ + 5% CO₂. Results are a summary of data obtained from Figs. 1 and 2.

<table>
<thead>
<tr>
<th>3-O-methyl-D-glucose uptake</th>
<th>In cells from starved animals</th>
<th>In cells from fed animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-O-methyl-D-glucose in extracellular medium (mM)</td>
<td>Initial rate (nmol/mg dry intracellular wt./min)</td>
<td>Conc. in space (mM)</td>
</tr>
<tr>
<td>5.0</td>
<td>18.0</td>
<td>3.8*</td>
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<tr>
<td>10.0</td>
<td>28.4</td>
<td>7.9*</td>
</tr>
<tr>
<td>15.0</td>
<td>47.6</td>
<td>11.4*</td>
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</table>

*Using an intracellular water space of 1.3 μl/mg dry wt.

Table 2. The effects of N₂-induced anoxia and KCN on the hexose-transport process of cells prepared from 24-h-starved and fed animals

Hepatocytes were prepared from fed and 24-h-starved rats. Cells were pre-incubated in a shaking water bath at 37°C, gassed with 95% O₂ + 5% CO₂, and, for N₂-induced anoxia, switched to 95% N₂ + 5% CO₂ for 5 min prior to and during uptake measurements, which were carried out at 22°C. In the case of respiratory inhibition, KCN was added to the cell suspension to a final concentration of 5 mM and incubated for 1 min at 22°C prior to uptake studies with 3-O-methyl-D-glucose. Type-I analyses are the means ± S.E.M. and the numbers of observations are in parenthesis.

<table>
<thead>
<tr>
<th>Type of analysis</th>
<th>Control</th>
<th>N₂-induced anoxia</th>
<th>KCN (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vₘₐₓ</td>
<td>Kₘ</td>
<td>Vₘₐₓ</td>
</tr>
<tr>
<td>Hexose transport in cells prepared from 24-h-starved animals</td>
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</tr>
<tr>
<td>I</td>
<td>161±12</td>
<td>39±4</td>
<td>108±15#</td>
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<tr>
<td></td>
<td>(7)</td>
<td>(3)</td>
<td>(3)</td>
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<tr>
<td>II</td>
<td>168</td>
<td>45</td>
<td>108</td>
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<td>(7)</td>
<td>(3)</td>
<td>(3)</td>
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<td>Hexose transport in cells prepared from fed animals</td>
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<tr>
<td>I</td>
<td>285±12#</td>
<td>54±3#</td>
<td>141±18*</td>
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<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td>II</td>
<td>270</td>
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<td>175</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
<td>(4)</td>
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</tbody>
</table>

Vₘₐₓ values are expressed as nmol/mg dry wt./min and Kₘ values are expressed as mM.

# P < 0.05 (as assessed by Student's t-test) compared to starved control.

* P < 0.05 (as assessed by Student's t-test) compared to fed control.
of 3-O-methyl-D-glucose in monolayer tissue cultures of hepatocytes (17) but not in the investigations of Baur and Heldt (3) or Craik and Elliott (4). Further evidence will be required to find a role, if any, for this phenomenon in vivo.

The effect of anoxia and KCN on the transport system

Cells prepared from fed animals show lowered kinetic parameters of the hexose-transport process when made anoxic; an even greater decrease is observed in the presence of KCN (Table 2). In the starved state anoxia also decreases the kinetic parameters observed in untreated cells but a surprising result is observed in the presence of KCN, where the $V_{\text{max}}$ and $K_m$ are increased nearly 3-fold and 2-fold respectively.

In both the fed and starved state anoxia causes a decrease in hepatic hexose transport. This is the opposite to the situation that occurs in muscle, where hexose transport is increased. KCN treatment of cells prepared from fed animals elicits an even greater decrease in hexose transport which would seem to be expected due to its complete inhibitory effect on oxidative metabolism. It is well known that in vivo the liver responds to hypoxia by utilizing glycogen and releasing glucose into the blood. Therefore, one might expect hexose transport into hepatocytes to be decreased when cells are made anoxic or exposed to KCN. The process by which this occurs has yet to be elucidated. However, KCN treatment of hepatocytes prepared from 24-h-starved animals produces an enormous increase in the kinetic constants of the transport system to values even higher than those obtained from untreated cells prepared from fed and starved animals. This result seems anomalous in view of the previously presented data, and will require further investigation for its explanation.

Acknowledgements

My thanks go to Professor Charles Pasternak and Dr. Patricia Whitton for their support and advice. I should also like to remember the late Dr. Douglas Hems for his advice and encouragement. This research was funded by the MRC.

References