Kinetics of 3-O-Methyl Glucose Transport in Red Blood Cells of Newborn Pigs

ROBERT B. ZEIDLER, PING LEE, and H. D. KIM

From the Department of Physiology and Biophysics, West Virginia University Medical Center, Morgantown, West Virginia 26505 and the Department of Physiology, University of Arizona School of Medicine, Tucson, Arizona 85724

ABSTRACT The glucose-permeable fetal red cells in the pig are entirely replaced by glucose-impermeable adult red cells within a month after birth. This study investigates the kinetic parameters of the glucose transport mechanism in newborn pig red cells in comparison with immature adult red cells (reticulocytes) as well as the fully matured adult erythrocytes. Influx and efflux of the nonmetabolizable 3-O-methyl glucose (3-O-M-G) in red cells of newborn pigs saturate at high substrate concentrations and exhibit typical Michaelis-Menten kinetics. $K_m$ values for efflux are 15.2 and 18.2 mM for 15 and 22°C, respectively. $Q_{10}$ computed between 10 and 26°C is 5.0. The energy of activation for the transport process is 34,000 cal mol$^{-1}$. The effectiveness of hexoses in competing with 3-O-M-G in efflux is in the following order: D-glucose > D-mannose > D-fructose > D-galactose. Efflux of 3-O-M-G does not increase with 3-O-M-G or D-ribose in the medium and is reduced by 2,4-dinitrofluorobenzene (DNFB), p-chloromercuriphenyl sulfonic acid (PCMBS), and phloridzin. The reticulocytes are shown to possess a carrier-mediated transport but with a considerably lower transport rate. As the reticulocytes mature into normal red cells, the carrier transport mechanism is lost.

INTRODUCTION

The transfer of glucose across the plasma membrane of red blood cells obtained from fetal animals is much greater than the transfer which occurs in red blood cells obtained from adult animals (Kozawa, 1914; Widdas, 1955). An outstanding example of this phenomenon occurs in pig red cells. Red cells from newborn piglets are highly permeable to glucose, while the red cells from adult pigs are nearly impermeable to glucose. As a consequence, the adult pig red cell is unable to utilize glucose and the energy source used in vivo is not known (Kim and McManus, 1971a, b).

The present study was undertaken with two objectives in mind. The first was to characterize the glucose transfer process in fetal pig red blood cells. Since this process is lost in red cells from the adult pig, it is of interest to know if this transport is any different from the glucose transfer process which occurs in the human red cells which are highly permeable to glucose at all stages of human maturation (Bowyer and Widdas, 1958; Karlish, 1972; Lacko et al., 1971; LeFevre, 1951; Levine and Stein, 1965; Widdas, 1953). Although Widdas (1955) indicated that a facilitated process may be present in fetal pig red blood cells,
kinetic data are not available. Secondly, since fetal blood is primarily comprised of cells of younger age, it is important to determine if the difference in membrane permeability to glucose between fetal and adult blood is due to the age difference of the cells. Hence, the glucose transport in reticulocytes produced in adult pig by phenylhydrazine injection was also studied.

In the present investigation a nonmetabolizable analog of glucose, 3-O-methyl glucose (3-O-M-G) was employed to examine the mechanism of glucose transfer. It was found that the transfer of 3-O-methyl glucose in red cells of newborn piglets is a carrier-mediated transport with extremely fast half time for equilibrium and that the reticulocytes in adult pig still retain the carrier-mediated transport mechanism but with a much slower equilibrium time. This carrier-mediated transport is lost when the reticulocytes mature to normal adult red cells.

**METHODS AND MATERIAL**

Fetal red cells were obtained from piglets born within 24 h when blood was taken. Blood was drawn by direct heart puncture into a syringe and coagulation was prevented by the use of 10 U of heparin for each milliliter of blood. The blood was centrifuged and the plasma and white buffy coat were removed. The red cells were washed four times in ice-cold 0.9% NaCl by alternate resuspending and centrifuging. Reticulocytes were obtained by producing reticulocytosis in a 150-lb pig with injection of 1 g of phenylhydrazine per day for a week.

The uptake of 3-O-methyl glucose in reticulocytes and adult pig cells was measured at 37°C. Since the transfer of glucose in fetal red cells is rapid at 37°C, the temperature at which influx and efflux are measured was lowered to 22°C. For efflux measurement a rapid sampling technique using a Millipore filter was employed (Mawe and Hempling, 1965). Red cells were loaded to a specific concentration of 14C-labeled 3-O-M-G. After centrifuging and removing supernatant fluid, an aliquot of 20-µl packed cells was mixed into a 20-ml volume of a medium which contained 143 mM NaCl, 4.8 mM KCl, 9.35 mM Na HPO₄, 1.65 mM Na H₂PO₄ at a pH of 7.4. Efflux is measured as the rate of appearance of labeled 3-O-M-G in the medium, by analysis of an accumulation compartment (Atkins, 1969). The modified equation which describes this accumulation of [14C]3-O-M-G is

\[
\ln\left(1 - \frac{S(t)}{S_\infty}\right) = -kt,
\]

where \(S(t)/S_\infty\) is the ratio of activity in the medium at time \(t\) over the activity in the medium at time infinity and \(k\) is the rate constant and \(t\) is time. The rate constant is determined from the slope of the line when \(\ln\left(1 - \frac{S(t)}{S_\infty}\right)\) vs. \(t\) is plotted.

The flux of 3-O-M-G is calculated by multiplying the internal 3-O-M-G concentration by the rate constant. Intracellular concentrations of sugar were computed by determining the amount of 14C label initially present in the cells. Influx measurement was carried out by incubating 1 ml of washed red cells in 10-ml media containing various 3-O-methyl glucose concentrations and electrolytes of essentially similar composition to the medium used for efflux measurement. [3-O-methyl¹⁴C] glucose was added to serve as tracer and ³H inulin was used as an extracellular tag. At different times after incubation at 22°C a 0.7-ml aliquot sample was removed and squirted into 7 ml of ice-cold 0.9% NaCl solution with 1 mM HgCl₂. The low temperature and HgCl₂ served to stop further influx of 3-O-methyl glucose and samples within 2-3 s may be taken. Quick sampling is necessary because of the fast transport of 3-O-methyl glucose in fetal red cells. The cold suspension was then quickly spun in a refrigerated centrifuge (Sorvall RC-2B, Dupont Instruments, Sorvall Operations, Newtown, Conn.) and the supernatant (SUP) solution was decanted. Red cells were then hemolyzed by adding 1 ml of distilled water and deproteinized by
procedures using either perchloric acid extraction or Ba(OH)₂ and Zn(SO₄). The latter
deproteinization procedure was accomplished by placing 0.2 cm³ of a sample into 2.0 cm³
of water. A volume of 0.2 cm³ of 0.3 M Ba(OH)₂ was added, and this suspension was
heated in a water bath at 60°C for 5 min. After 5 min, 0.2 cm³ of 5% ZnSO₄ was added and
the suspension vortexed. After centrifuging, aliquots of clear supernatant solution were
used in liquid scintillation counting. For perchloric acid extraction, 0.2 cm³ of a sample
was placed into 2.0 cm³ of 3.5% perchloric acid, vortexed, then centrifuged. To 1.5 cm³ of
the supernatant solution was added 0.1 cm³ of 5.6 mM K₂CO₃ in order to neutralize the
acid. Aliquots of the clear supernatant were used in counting the radioactivity. Since it
has been shown that while the Ba(OH)₂-Zn(SO₄)₄ procedure results in removal of phospho-
rylated sugar, the PCA extraction does not (Thomas et al., 1970), red cells were extracted
by both methods in order to determine whether 3-O-methyl glucose was phosphorylated.
Table I shows the results of a 3-O-methyl glucose uptake experiment using the two
different deproteinization procedures for extraction of tracer. The activities in cell by
Ba(OH)₂-Zn(SO₄)₄ procedure were lower than those by PCA method. However, because
of the scatter of the data, these differences are not considered significant. It is important
to note that the ratios of activities in and out of the cells were close to 1.0 indicating that
the sugar was in equilibrium after 2 h of incubation. If there was any phosphorylation by
the hexokinase reaction at all, the amount phosphorylated must be very small indeed.

| Table I |
| UPTAKE OF ¹⁴C-LABELED 3-O-METHYL GLUCOSE BY RED BLOOD CELLS OF A 1-DAY-OLD PIGLET |
| | Perchloric acid | Barium hydroxide zinc sulfate |
| Hours incubation | 2 | 6 |
| Activity* inside cells | 183 | 200 | 146 | 176 |
| Activity* in medium | 186 | 174 | 186 | 174 |
| Activity* in cell/ activity* in medium | 1.0 | 1.1 | 0.8 | 1.0 |

* Activity = [¹⁴C]3-O-M-G cpm/ml.
Red cells were incubated with 10.0 mM 3-O-M-G at 22.0°C for 6.0 h. Then they were deproteinized
using perchloric acid or barium hydroxide and zinc sulfate.

3-O-Methyl Glucose Uptake in Red Cells of Pigs

The 3-O-methyl glucose (3-O-M-G) uptake in red cells of pigs shows drastic
changes as the pigs grow older. Fig. 1 shows the rate of 3-O-M-G uptake in red
cells from growing pigs relative to the initial value obtained at birth. In 30 days
after birth the glucose permeability has already been reduced to that of adult
level.
The influx rate of 3-O-methyl glucose in red cells from 1-day-old piglets was very high. Half time of equilibrium when external concentration is 1–5 mM is between 20–30 s. The initial 15-s flux rate (Fig. 2) is seen to be dependent on external 3-O-M-G concentrations, especially at lower substrate concentration;

![Graph](image)

**Figure 1.** 3-O-Methyl glucose uptake in red cells from growing pigs. Uptake rates given are relative to the rate obtained in red cells from 1-day-old pigs.

![Graph](image)

**Figure 2.** 3-O-Methyl glucose uptake as a function of external 3-O-M-G concentrations. Measurements were carried out at 22°C. Insert shows the Lineweaver-Burk plot of the same data.

the increment in flux rate is smaller at higher external concentration, indicating a saturation type of transport. The influx does not plateau completely at higher external concentration, instead it increases linearly with a much smaller slope. This indicates that there is also a nonsaturable component (probably diffusion) in addition to a saturable system. A Lineweaver-Burk plot (Fig. 2, right panel)
yields a $K_m$ (sugar concentration at which one-half of the maximal transport rate occurs) of 23 mM and a $V_{max}$ (maximal transport rate) of 27 $\mu$mol/ml red blood cells (RBC)/min.

**Characteristics of 3-O-M-G Efflux**

The efflux of 3-O-M-G is characterized by a two-compartment system. This is seen in Fig. 3 A when an intracellular concentration of 18.8 mM 3-O-M-G is present and in Fig. 3 B when intracellular concentration is 43.5 mM 3-O-M-G. In each case the flux is represented by a large initial linear fast portion followed by a smaller slow portion. The initial fast portion is linear and represents the majority of the efflux and the slope of this line is used as a measure of the rate constant of 3-O-M-G efflux for computation of flux rate given in the following sections.

**Figure 3.** Efflux of $^{14}$C-labeled 3-O-methyl glucose from red blood cells of 1-day-old piglet. $S_t/S_\infty$ is the ratio of $^{14}$C activity in the efflux medium at time ($t$) and the activity at time infinity ($\infty$). Panel A shows the efflux when the cells were loaded with 18.8 mM 3-O-M-G and panel B shows the efflux when the cells were loaded with 43.5 mM 3-O-M-G. Each efflux can be resolved into a two-compartment system represented by the dashed lines. The equation describing the two compartment efflux shown in panel A is $Y = 0.58e^{-1.40t} + 0.12e^{-0.07t}$. The equation for the efflux seen in panel B is $Y = 0.05e^{-0.32t} + 0.13e^{-0.07t}$. 

Published January 1, 1976
The net 3-O-M-G efflux is dependent on internal 3-O-M-G concentration. Fig. 4 shows the results of measurements of the flux rate at 22 and 15°C. As the internal 3-O-M-G concentration increases, the flux rate increases and then reaches a plateau. This feature is consistent with Michaelis-Menten kinetics.

By replotting the data in a Lineweaver-Burk plot (Fig. 4 top right corner), $V_{\text{max}}$ is found to be 58.8 μmol/ml RBC/min and $K_m$ is 18.2 mM. These numbers are somewhat different from those found for influx, but it is doubtful that the difference is due to a significant asymmetry of the transport system. Rather, it is more likely that this arises from the different procedures of measuring influx and efflux. It is important to note that both influx and efflux show saturation kinetics.

**Apparent Energy of Activation**

The demonstration of saturable efflux of 3-O-M-G in the previous section suggests that a carrier-mediated mechanism is involved. It is assumed here that
for the sugar molecule to cross the red cell membrane, it must react with the transport carrier; therefore, an apparent energy of activation for the transport process may be determined. To ensure that no secondary reactions are involved in this process, an Arrhenius plot was determined between the temperatures of 26 and 10°C (Fig. 5). Single linear results were found when the log of the rate constant was plotted against the reciprocal of the temperature indicating that no secondary reactions are interfering with the determination of the apparent activation energy. The energy of activation for the transport process was determined for several 3-O-M-G concentrations at 15 and 22°C. The results are shown in Table II. The average energy of activation was 34,600 cal mol⁻¹.

**Figure 5.** Effect of temperature on the efflux rate constants of 3-O-methyl glucose from red blood cells of a 1-day-old piglet. The logarithm of the rate constant is plotted against the reciprocal of the temperature (°K).

**Competition for Efflux**

In order to examine the specificity of the transport carrier, efflux of 3-O-M-G was measured in the presence of other structurally related sugars. The red cells were loaded with 40.0 mM 3-O-M-G and 40.0 mM of another sugar for 2–3 h to allow for equilibrium of sugar to occur and the efflux of 3-O-M-G was measured. If a particular substrate shares the same carrier with 3-O-M-G, then it will compete with 3-O-M-G for transport. A decrease in the efflux rate of 3-O-M-G is expected, the extent of the decrease depends on the relative affinity of the carrier for these two sugars. D-glucose, D-galactose, D-mannose and D-fructose were employed in this competition study. The results are shown in Fig. 6. Galactose produces a 10% inhibition in 3-O-M-G efflux, fructose 14%, mannose 27%, and glucose 43%. Note that the 3-O-M-G efflux is decreased almost by one-
half by D-glucose; furthermore, addition of 40 mM D-glucose to 40 mM 3-O-M-G results in an efflux rate constant nearly equal to the rate constant when red cells were loaded with 80 mM 3-O-M-G (Table III). This indicates that the affinity of the carrier for 3-O-M-G and D-glucose is nearly the same.

**TABLE II**

ENERGY OF ACTIVATION GLUTOSE-TRANSPORT COMPONENT COMPLEX

<table>
<thead>
<tr>
<th>Internal 3-O-methyl glucose concentration</th>
<th>Energy of activation cal mol⁻¹⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.69</td>
<td>38,600</td>
</tr>
<tr>
<td>18.8</td>
<td>32,700</td>
</tr>
<tr>
<td>43.5</td>
<td>34,500</td>
</tr>
<tr>
<td>73.5</td>
<td>32,400</td>
</tr>
<tr>
<td>Average</td>
<td>34,600</td>
</tr>
</tbody>
</table>

The apparent activation energy (Eₘ) for the transport process was determined from the ratio of efflux rates of 3-O-M-G at 22 and 15°C using the Arrhenius equation:

\[
E_a = \frac{4.56T_1 T_2}{T_2 - T_1} \log \frac{V_2}{V_1}
\]

**Figure 6.** The effect of hexoses on the efflux of 3-O-methyl glucose from red blood cells of a 1-day-old piglet. Red cells were loaded with 40.0 mM 3-O-M-G and 40.0 mM of another sugar and efflux into a sugar-free medium was measured. The efflux rate constant of 3-O-M-G from cells loaded with only 40.0 mM 3-O-M-G is taken as control which is denoted as 1.0. Values plus range from four experiments are plotted and all values are significantly different from the control value (P < 0.05 as determined by the t test of E. Lord (1947)).
Inhibition by Drugs

If the transport of 3-O-M-G depends upon a particular configuration of the transport component, it would be possible to attach specific groups on the component and disrupt the configuration thus altering the efflux of 3-O-M-G. Newborn pig red cells were loaded with 40 mM 3-O-M-G and the 3-O-M-G was allowed to efflux into a medium which contained a specific drug. The concentrations were 1.0 mM, 2, 4-dinitrofluorobenzene (DNFB), 2,4,6-trinitrobenzene sulfonic acid (TNBS) N-ethylmaleimide (NEM), urethane, and phloridzin. A concentration of 0.1 mM p-chloromercuriphenyl sulfonic acid (PCMBS) was also employed. The results are shown in Fig. 7. TNBS, NEM, and urethane have no effect, while DNFB produces 36% inhibition, PCMBS an 82% inhibition, and phloridzin an 85% inhibition.

Efflux of 3-O-M-G into a Medium Containing 3-O-M-G or Ribose

The transfer of 3-O-M-G implies that the membrane transport component must have access to both sides of the membrane. Conceivably another sugar present in the efflux medium may attach to the transport component. If this happens, a change in efflux rate might be expected, depending on whether the loaded carrier component is more mobile than the free nonloaded carrier. Piglet red cells were loaded with a concentration 20 or 50 mM 3-O-M-G. Efflux rates of 3-O-M-G into a media which contains no sugar, 20, 40, 60, or 80 mM 3-O-M-G were measured. Media containing no sugar, 20, 40, or 80 mM ribose were also tested. Relative efflux rates were determined by dividing the experimentally determined rate constant found when no sugar was in the external medium, into the rate constant found when sugar was present in the external medium. The results shown in Fig. 8 indicate that neither 3-O-M-G nor ribose in external medium affect the efflux of 3-O-M-G.

Counter Transport

Although newborn cells do not seem to show exchange flux, it was possible to demonstrate the existence of counter transport in these cells. When glucose was

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**TABLE III**

<table>
<thead>
<tr>
<th>Loaded sugar concentration</th>
<th>Rate constant (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.0 mM 3-O-M-G</td>
<td>1.25</td>
</tr>
<tr>
<td>80.0 mM 3-O-M-G</td>
<td>0.70</td>
</tr>
<tr>
<td>40.0 mM 3-O-M-G</td>
<td>0.66</td>
</tr>
<tr>
<td>40.0 mM D-Glucose</td>
<td></td>
</tr>
</tbody>
</table>
added to the medium after 14C-labeled 3-O-M-G had reached equilibrium, the 3-O-M-G labels in medium increased, then fell back to its original level (Fig. 9). This observation is consistent with the concept of carrier-mediated transport existing in these cells.

3-O-Methyl Glucose Transport in Reticulocytes and Adult Pig Red Cells

Fig. 10 shows the 3-O-methyl glucose influx in reticulocytes and mature red cells from adult pig. Because of the slow uptake rate of 3-O-methyl glucose in reticulocytes and mature adult red cells, measurements were carried out at 38°C instead of 22°C which influx measurement of 3-O-M-G in newborn cells were performed. Influx of 3-O-methyl glucose in reticulocytes is concentration dependent at low substrate concentrations but tends to saturate at higher substrate concentrations. This indicates that reticulocytes retain a carrier-mediated transport mechanism although the transport rate is much slower than that found in newborn cells. When the reticulocytes lose their reticulum and mature to normal adult cells, the carrier-mediated transport mechanism is lost. In contrast to reticulocytes, the influx of 3-O-M-G in the adult red cells is a concentration-dependent first-order transport process.

DISCUSSION

The results presented above clearly show that a carrier-mediated mechanism is involved in the 3-O-methyl glucose transport in red cells of newborn pigs. In this regard, this investigation confirms and extends the initial observation by Widdas (1955) who postulated the carrier mechanism for fetal pig red cells.
Although the overall transport characteristics in piglet red cells are not unlike those found in human red cells (Widdas, 1953; LeFevre and Davies, 1951; LeFevre and McGinness, 1960) and rabbit red cells (Regen, 1964), there are several distinct differences which are worthy to note. The transport rate in piglet red cells is much slower than that found for human red cells. At 22°C the maximal transport rate ($V_m$) in piglet red cells is about one-third that for adult human red cells (Mawe and Hempling, 1965) but $K_m$ value (the substrate concentration at which the flux rate is one-half maximal) is similar to those found for net fluxes in human red cells (Karlish et al., 1972). It seems that the transport mechanism for glucose in those two cell types shows similar affinities for glucose but since the maximum flux rate is smaller in piglet red cells, the latter may have fewer transport sites (or carriers) or the turnover rate (mobility of carrier) is slower. The very high activation energy, 34,600 cal mol$^{-1}$ and the high $Q_{10}$ value (5 as compared to 2–3 found for human red cells), may suggest that the rate-limiting steps in the transport are probably (a) at the site of reaction where sugar binds with the carrier, and (b) the translocation of the sugar-carrier.

**Figure 8.** Efflux of 3-O-methyl glucose from red blood cells of a 1-day-old piglet into a medium containing 3-O-M-G or ribose. Panel A shows results obtained from two animals when red cells were loaded with 20.0 or 50.0 mM 3-O-M-G and effluxes into an external media containing no sugar, 20.0, 40.0, 60.0, or 80.0 mM 3-O-M-G were determined. Panel B shows the results from two animals when these red cells were placed in a medium which contained no sugar, 20.0, 40.0, or 80.0 mM ribose and efflux rates were determined.
complex from one side of the membrane to the other. The similarity $K_m$ values found for piglet and human red cells suggests that the magnitude of the energy barrier involved in substrate-carrier combination is approximately the same. Hence, the difference in rate of transport is most likely to be due to the translocation of the sugar-carrier complex. It is possible that this translocation in piglet red cells is more sensitive to temperature change than that in human red cells.

Another important difference between newborn pig red cells and human red cells is that in adult human red cells, the rate of efflux of a sugar into a medium which contains sugar is two to three times faster than the rate of efflux of a sugar into a sugar-free medium (Lacko and Berger, 1963; Levine and Stein, 1965; Mawe and Hempling, 1965). In newborn pig red cells, no enhancement of fluxes is shown with sugar present in the external medium. This is similar to results found in adult rabbit red cells (Regen, 1964) and bovine red cells (Hoos et al., 1972). Therefore, it seems reasonable to conclude that the loaded carrier moves no faster than the unloaded carrier in newborn pig red cells.
It is of interest to note that when the pig matures, somehow its red cells no longer possess this part of the transport machinery. It is unlikely that the difference found between newborn red cells and adult red cells is due to differences in cell ages. The reticulocytes still retain a carrier-mediated transport for 3-O-methyl glucose which is lost when the cells mature. The magnitude of this transport, however, is at least two or three orders of magnitude lower than that found in newborn cells. Therefore, there seem to be basic differences between fetal cells and adult cells. Whether this is due to a loss of this component in the red cells produced after birth or to a masking effect owing to the presence of other materials is not known at this time.

As a consequence of this loss of the membrane permeability to glucose, pig red cells become incapable of utilizing glucose for the production of necessary free energy to maintain their cellular integrity (Kim et al., 1973; Kim and McManus a, b, 1971). Despite this metabolic anomaly, the postnatal right-hand shift of oxygen hemoglobin dissociation curve often found in newborn mammals still takes place in the pig mainly due to a rapid rise of red cell 2,3-DPG content (Kim and Duhm, 1974). Clearly, the transition from glycolytic fetal red cells to nonglycolytic adult red cells has little bearing upon the red cells' primary role of delivering oxygen to the tissue. The intriguing problem of how nonglycolytic mammalian red cells can survive in circulation awaits further studies.

This study was supported by NIH grant HL-13237 to P. Lee and NIH grant AM-17723 and S. Arizona Heart Grant to H. D. Kim.

Part of this work appears in a dissertation submitted to West Virginia University by R. B. Zeidler in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Received for publication 10 September 1974.

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