Metabolic Control of Sugar Transport by Derepression of Cell Surface Glucose Transporters

AN INSULIN-INDEPENDENT RECRUITMENT-INDEPENDENT MECHANISM OF REGULATION

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Protein-mediated sugar transport is nominally absent in normoxic pigeon erythrocytes. Following exposure to metabolic inhibitors (cyanide or carbonylcyanide-p-trifluoromethoxyphenylhydrazone), pigeon red cells transport sugars by a saturable stereoselective pathway that is inhibited by cytochalasin B or forskolin. The sugar transport capacity of fully poisoned cells is consistent with a transporter density of approximately 30 carriers/erythrocyte. Immunoblot analyses and competition enzyme-linked immunosorbent assay indicate that pigeon red cells contain approximately 200 copies of an integral plasma membrane protein immunologically related to the glucose transporter isoform GLUT1. GLUT1 is quantitatively restricted to the plasma membrane at all times. Pigeon red cells and brain lack proteins immunologically related to the glucose transporter; GLUTB, human fetal skeletal muscle glucose transporter; GLUT4, rat skeletal muscle insulin-regulated glucose transporter; GLUT5, rat skeletal muscle insulin-regulated glucose transporter.

The facilitated diffusion of sugars (sugar transport) by striated muscle is stimulated massively during exposure to insulin and catabolic conditions that lead to increased demand for glycolytic ATP (exercise, anoxia, and inhibition of oxidative phosphorylation) (1, 2). These transport stimulations appear to coincide with a doubling of sarcocoelemmal sugar transporter content (3–7), but the mechanisms mediating insulin and metabolic regulation of transport are fundamentally different (8–10). Thus insulin stimulation of sugar transport is blocked in both metabolically depleted adipose (8) and muscle (11). However, basal (insulin-independent) transport in adipose tissue is unchanged by metabolic depletion (8), whereas skeletal muscle basal sugar transport is stimulated massively by metabolic depletion (1, 2). These observations suggest that muscle expresses a unique regulatory mechanism that mediates transport stimulation when cellular demand for glycolytic ATP is increased.

We have chosen to study metabolic regulation of sugar transport in avian red cells for a number of reasons. Sugar transport by nucleated (avian) erythrocytes is stimulated by metabolic depletion and hypoxia but not by insulin (12–17). Avian erythrocytes are devoid of significant intracellular structures other than mitochondria and the nucleus (18, 19), are characterized by vanishingly low rates of transcription (20), are unable to export nuclear RNA (20, 21), are devoid of cytoplasmic RNA, and are protein synthesis-incompetent (22, 23). These cells thus provide a unique opportunity to study insulin-independent metabolic regulation of sugar transport in the absence of a background of de novo protein synthesis and constitutive membrane recycling. Available in suspension and amenable to lysis/resealing procedures, these cells also permit a level of transport analysis that is not possible using striated muscle.

In this study we demonstrate that protein-mediated sugar transport is nominally absent in freshly drawn pigeon erythrocytes (see also Ref. 16) but that following metabolic depletion, pigeon erythrocytes transport sugars by a saturable stereoselective mechanism that is inhibited by cytochalasin B and forskolin. We further show that transport is mediated by a GLUT1-like sugar transport protein and that sugar transport stimulation by metabolic depletion results from derepression of cell surface sugar transport proteins.

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EXPERIMENTAL PROCEDURES

Materials—[U-14C]3-O-Methylglucose, [U-14C]glucose, [H]cytochalasin B, and [125I]protein A were purchased from Du Pont- New England Nuclear. 3-[[126I]iodo-4-azidophenethylamido-7-O-succinyldiacetylforskolin ([126I]APS-forskolin) was a generous gift from M. F. Shanahan. [3H]2-N-[4(1-Azi-2,2,2-trifluoromethyl)benzoyl]-1,3-bis-(O-mannos-4-yloxy)-2-propylamine ([3H]ATB-BMPA) was a generous gift from G. D. Holman. All other reagents were purchased from Sigma unless indicated otherwise. Anti-GLUT1, anti-GLUT3, and anti-GLUT4 antisera were purchased from East Acres Biologicals. Male Silver King pigeons (5 years old) were purchased from the Palmetto Pigeon Plant (Sumter, South Carolina). Blood was obtained from each anesthetized pigeon by cervical dislocation and collection into 20 ml of trisodium citrate (75 mM, pH 7.4, 24 °C). Whole outdated

* The abbreviations used are: GLUT1, human erythrocyte glucose transporter; GLUT3, human fetal skeletal muscle glucose transporter; GLUT4, rat skeletal muscle insulin-regulated glucose transporter; ATB-BMPA, 2-N-[4(1-azi-2,2,2-trifluoromethyl)benzoyl]-1,3-bis-(O-mannos-4-yloxy)-2-propylamine; IAPS-forskolin, 3-iodo-4-azidophenethylamido-7-O-succinyldiacetylforskolin; FFCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; PMSF, phenylmethylsulfonyl fluoride; ß-IGF, rabbit anti-GLUT1 exofacial epitope antisera; C-IGG, rabbit anti-GLUT1 COOH-terminal peptide antisera; C2-IGG, rabbit anti GLUT1 antisera; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; Ht, hematocrit.
human blood was obtained from the University of Massachusetts Medical Center Blood Bank. Frozen brain and spleen from pigeons were purchased from Rockland (Gibertsville, PA).

**Preparation of Avian Erythrocytes and Erythrocyte Ghosts—**Pigeon red cells, collected in citrate, were filtered through cheese cloth to passage through a 26-gauge needle. The final suspension (1-16 mg of protein/ml) was collected, and the pellet (containing largely proteins and lipid) was washed and centrifuged at 23,300 g. In some experiments, 1 mg of protein/ml was added. Solubilization buffer contained 150 mM KCl, 5 mM Tris-HE, 0.2 mM EDTA, pH 7.4 (24°C). The supernatant was discarded. The resuspended ghosts were frozen at -20°C overnight lyophilized to 3 mg of protein/ml and stored at -70°C.

**Isolation of Erythrocyte Integral Membrane Proteins—**Integral membrane proteins were solubilized from pigeon erythrocyte ghosts using 0.5% Triton X-100 (25, 26). Ghosts were resuspended in 5 volumes of ice-cold Triton buffer by gentle mixing and then placed on ice for 30 min. The suspension was centrifuged for 3 min at 24,000 g. The supernatant was removed, the cells were resuspended in 5 volumes of ice-cold medium, and the suspension was subjected to five additional wash centrifugations. The final red cell ghost pellet was carefully decanted into 20 volumes of lysis medium containing 0.3 M sucrose, leaving a deep red button of lysate-resistant cells which was discarded. The resuspended ghosts were frozen at -20°C overnight and then thawed the following morning and subjected to an additional three cycles of wash centrifugation in lysis medium. At this time, the ghosts were substantially free of hemoglobin, retained an erythroid shape, and contained intact nuclei. Unsealed human erythrocyte ghosts were prepared from washed intact human erythrocyte ghosts by using an anti-GLUT1 antibody (e.g., 600 mM 3-O-methylglucose), control media also contained 600 mM L-glucose or sorbitol. When cytochalasin B (100 μM) was used to inhibit label incorporation, control media also contained carrier (dimethyl sulfoxide) at an appropriate concentration. Photolysis with [125I]lodoactosamine for 1 min at 300 nm using a Rayonet photo reactor and was carried out as described by Wardzinski et al. (33) using a 1-cm path length quartz cuvette. Irradiated ghosts were collected and washed in 10 ml of 0.5 mM sodium phosphate, 4 mM MgCl₂, pH 8.0, 1% β-mercaptoethanol ± inhibitors present prior to irradiation. Ghosts were sedimented and washed an additional 3 times in 10 min. The suspension was centrifuged for 18 h at 4°C. Protein A-Sepharose beads (60 μl of a 50% suspension in solubilization buffer) were added, and the suspension was incubated for an additional 3 h at 4°C by end-over-end rotation. At this time, the protein A-Sepharose beads were sedimented, and the supernatant was reconstituted with 30 mg of egg phosphatidylcholine by cholate dialysis (28, 29). n-Glucose (20 mM) uptake was monitored at 24°C with resuspending 1 volume proteoliposomes (±10 min preincubution in 20 mM cytochalasin B) in 1 volume of uptake medium (cholate-free solubilization buffer) containing unlabeled and [14C]glucose. At 0, 1, or 5 min 500 μl of stopper (cholate-free solubilization buffer containing 250 μM phloretin and 25 μM cytochalasin B at ice temperature) were added, and the suspension was centrifuged for 5 min at 14,000 g at 4°C. The supernatant was then incubated, and the pellet was dissolved in cholate-free solubilization buffer containing 0.5% Triton X-100. Aliquots were counted by liquid scintillation spectrometry, and uptake was computed as counts/min/mg [14C]glucose associated with proteoliposomes at time t minus counts/min/mg [14C]glucose associated with proteoliposomes at zero time.

**Ligand Binding Assays—**[125I]Cytochalasin B binding measurements were performed as described in Refs. 30 and 31.

**Affinity Labeling the Glucose Transporter—**Human erythrocyte and pigeon red cell glucose transporters were photolabeled using either [125I]lodoactosamine or [3H]ATB-BMPA. The method was an adaptation of that employed in [125I]cytochalasin B photolabeling experiments (28, 32). Human or pigeon red cell ghosts were resuspended to a final Ht of 40% in lysis medium containing 1 mM [125I]lodoactosamine (0.185 μCi/μl) or 70 μM [3H]ATB-BMPA (10 Ci/mmol). We have reported suggested inhibition, and the pellet was dissolved in cholate-free solubilization buffer containing 0.5% Triton X-100. Aliquots were counted by liquid scintillation spectrometry, and uptake was computed as counts/min/mg [14C]glucose associated with proteoliposomes at time t minus counts/min/mg [14C]glucose associated with proteoliposomes at zero time.

**Derepression of Cell Surface GLUT1—**Pigeon erythrocyte glucose transporter proteins (1 mg in 0.5 ml of cholate-free solubilization buffer) were resuspended in solubilization buffer containing cholate. The suspension was centrifuged at 4°C for 30 min and the supernatant was collected and then centrifuged at 31,000 × g for 30 min at 4°C. The supernatant (600 μl) was then incubated by end-over-end rotation with either 15 μl of preimmune rabbit serum or with 15 μl of anti-GLUT1 antisera for 18 h at 4°C. Protein A-Sepharose beads (60 μl of a 50% suspension in solubilization buffer) were added, and the suspension was incubated for an additional 3 h at 4°C by end-over-end rotation. At this time, the protein A-Sepharose beads were sedimented, and the supernatant was reconstituted with 30 mg of egg phosphatidylcholine by cholate dialysis (28, 29). n-Glucose (20 mM) uptake was monitored at 24°C with resuspending 1 volume proteoliposomes (±10 min preincubation in 20 mM cytochalasin B) in 1 volume of uptake medium (cholate-free solubilization buffer) containing unlabeled and [14C]glucose. At 0, 1, or 5 min 500 μl of stopper (cholate-free solubilization buffer containing 250 μM phloretin and 25 μM cytochalasin B at ice temperature) were added, and the suspension was centrifuged for 5 min at 14,000 g at 4°C. The supernatant was then incubated, and the pellet was dissolved in cholate-free solubilization buffer containing 0.5% Triton X-100. Aliquots were counted by liquid scintillation spectrometry, and uptake was computed as counts/min/mg [14C]glucose associated with proteoliposomes at time t minus counts/min/mg [14C]glucose associated with proteoliposomes at zero time.

**Immuno blot Analyses—**Western blot analyses were carried out as described previously (24, 32, 34). In some experiments, cross-reactivity of an avian erythrocyte protein of heterogeneous electrophoretic mobility and average mass of 48 kDa was detected by using an anti-GLUT1 antibody. In some experiments, the autoradiographic density (28, 32). Human or pigeon red cell ghosts were resuspended to a final Ht of 40% in lysis medium containing 1 mM [125I]lodoactosamine (0.185 μCi/μl) or 70 μM [3H]ATB-BMPA (10 Ci/mmol). We have reported suggested inhibition, and the pellet was dissolved in cholate-free solubilization buffer containing 0.5% Triton X-100. Aliquots were counted by liquid scintillation spectrometry, and uptake was computed as counts/min/mg [14C]glucose associated with proteoliposomes at time t minus counts/min/mg [14C]glucose associated with proteoliposomes at zero time.

**In vitro** uptake of 3-O-methylglucose was measured in the presence of control experiments on GLUT1 also included the carrier (dimethyl sulfide) at the appropriate concentration (0.5% by volume).
and preincubated with exogenous tetrameric GLUT1 (0–20 μg), human erythrocytes or unsealed ghosts (0–10⁶ cells), or with pigeon red cells or unsealed red cell ghosts (0–2 × 10⁵ cells) for 2 h at 24 °C by end-over-end rotation. Cells, ghosts, and GLUT1 were removed by centrifugation, and aliquots of the supernatant were assayed for tetrameric GLUT1 binding.

Other Assays—Protein was quantitated by the BCA assay (Pierce Chemical Co.). SDS-PAGE (10% acrylamide) was as described in (35). Erythrocytes were counted by using a hemocytometer.

**RESULTS**

3-O-Methylglucose Uptake by Pigeon Erythrocytes—Fig. 1A shows a representative experiment in which the time course of 3-O-methylglucose uptake at 0.8 mM sugar was measured in control cells at 37 and 4 °C and in cells poisoned with 1 mM NaCN plus 4 μg·ml⁻¹ FCCP for 2 h at 37 °C prior to uptake measurements at 37 or at 4 °C. Uptake by control and poisoned cells at 37 °C follows an exponential time course characterized by rate constants, k, of 0.0058 and 0.0277 min⁻¹, respectively. Transport stimulations by NaCN and FCCP (5-fold) are indistinguishable. The equilibrium 3-O-methylglucose spaces of control and poisoned cells (90 fl/cell) are indistinguishable and are not significantly different from the water content of the erythrocyte (88 fl/cell (16)). Relative to uptake by control and poisoned cells at 37 °C, uptake by control cells at ice-temperature is significantly reduced. Assuming that the free water contents of cells at 4 °C and 37 °C are identical, k for uptake on ice is 66.7 × 10⁻⁶ min⁻¹ which represents a 90-fold reduction in the rate of control sugar uptake.

These results indicate that sugar uptake by avian erythrocytes is passive (the equilibrium sugar space of the cell does not exceed the water content of the cell), is characterized by an activation energy of 20 kcal·mol⁻¹ (over the temperature range 4–37 °C), and is stimulated following exposure to metabolic poisons. These results do not, however, indicate whether sugar uptake occurs via protein-mediated or leakage pathways.

Fig. 1B illustrates a representative experiment in which the time course of sugar uptake stimulation (at 0.1 mM sugar) by FCCP was determined. Uptake was measured either in the presence or absence of 50 μM cytochalasin B (an inhibitor of protein-mediated sugar transport), and at zero time cells were exposed either to FCCP (4 μg·ml⁻¹) or carrier (0.1% ethanol) alone. Aliquots of the suspensions were sampled at various intervals and 3-O-methylglucose uptake was measured over a 10-min period. Based upon the results of Fig. 1A, uptake by control cells during a 10-min interval should approach 5.6% of equilibrium uptake, whereas uptake by poisoned cells should approach 24% of equilibrium uptake. This means that transport stimulation is underestimated at most by 10% by measuring uptake over a 10-min interval.

This figure shows that control sugar uptake is unaffected by incubation of cells at 37 °C for intervals of up to 210 min. Uptake in poisoned cells increases exponentially with duration of exposure to poison and reaches a maximum of approximately 5-fold greater than uptake by control cells with an apparent t₅₀ of 38 min. Whereas 50% of control cell sugar uptake is inhibited by cytochalasin B, 90% of total sugar uptake in fully poisoned cells is inhibited by cytochalasin B. This means that cytochalasin B-inhibitable 3-O-methylglucose uptake is stimulated 10-fold by metabolic poisoning. Moreover, transmembrane sugar leakage (cytochalasin B-control (C) and poisoned (D) cells).

The curves were computed by nonlinear regression assuming uptake is passive and stimulated by simple Michaelis-Menten kinetics and the results were:

- Control, Uₚ = 2.5 ± 0.01 μm·min⁻¹, Kₘₕₚ = 0.35 ± 0.07 mM, R = 0.7; poisoned cells, Uₚ = 55.4 ± 3.4 μm·min⁻¹, Kₘₕₚ = 3.4 ± 0.7 mM, R = 0.987.
insensitive sugar uptake) is unaffected by metabolic poisoning. The cytochalasin B levels used in this experiment are not limiting for inhibition of protein-mediated sugar uptake, since control experiments indicate that inhibitions of transport produced by 20–100 μM cytochalasin B are indistinguishable. In addition, cytochalasin B inhibits pigeon red cell protein-mediated sugar uptake with a $K_{\text{m(app)}}$ of 200 nM (16), thus 20 μM cytochalasin B should produce 99% inhibition of protein-mediated transport.

The concentration dependence of 3-O-methylglucose uptake by control and FCCP-poisoned cells is shown in Fig. 1C. Sugar uptake was measured over a 10-min interval in control cells and in cells exposed to FCCP for 2 h. These conditions underestimate stimulated uptake at low sugar levels ($<K_{\text{m(app)}}$) by approximately 10%. The net effect is that $K_{\text{m(app)}}$ for 3-O-methylglucose uptake in poisoned cells is overestimated by 14% but estimates of $V_{\text{max}}$ are accurate. Since exposure to poison for 2 h results in stimulation of sugar uptake to 90% of maximal values (see Fig. 1B), the maximum achievable $V_{\text{max}}$ is underestimated by 10%.

Fig. 1C shows that cytochalasin B-inhibitable 3-O-methylglucose uptake displays simple saturation kinetics in both control and poisoned cells. $V_{\text{max}}$ and $K_{\text{m(app)}}$ for sugar uptake by poisoned cells are 19- and 10-fold greater, respectively, than in control cells. These data are corrected for cytochalasin B-insensitive 3-O-methylglucose uptake which increases monotonically with $[3\text{-O-methylglucose}]$ in both control and poisoned cells. As in Fig. 1B, the magnitude of this component of 3-O-methylglucose uptake is not significantly affected by cellular metabolic depletion ($K_{\text{obs}} = 0.0039 \text{ min}^{-1}$). Thus, at 5 mM 3-O-methylglucose, cytochalasin B-inhibitable sugar uptake accounts for 14% and 66% of sugar uptake by control and poisoned cells, respectively. Table I summarizes the results of 3 similar experiments.

Cytochalasin B-inhibitable 3-O-methylglucose uptake by poisoned cells is also inhibited by maltose ($K_{\text{m(app)}} = 44 \pm 8.5 \text{ mM;} n = 3$) and by forskolin ($K_{\text{m(app)}} = 3.6 \pm 0.1 \mu\text{M;} n = 4$) but not by sucrose.

These features indicate that pigeon erythrocytes contain a saturable stereoselective transport pathway (sugar transporter) that is stimulated during metabolic poisoning and which resembles the human erythrocyte glucose transporter in terms of its stereoselectivity and susceptibility to pharmacologic inhibitors. These experiments do not indicate, however, whether transporter stimulation results from activation of cell surface sugar transporters or from recruitment of intracellular transporters to the plasma membrane.

**Transport Stimulation: Recruitment of Activation?**—Two strategies were used to determine whether cell surface glucose transporter (GLUT) density is altered by metabolic depletion of pigeon red cells. The first exploits the use of high affinity, photoreactive ligands that interact specifically with all characterized sugar transporters. These ligands include [125I]cytochalasin B, [125I]IAPS-forskolin, and [3H]ATB-BMPA. The second approach exploited immunological probes of cellular GLUT.

**Ligand Binding Sites**—Of the three photoaffinity ligands employed, one ([3H]ATB-BMPA) is cell membrane impermeant and reacts only with the sugar uptake site of the transporter in intact mammalian cells (36). The remaining ligands rapidly penetrate cells to interact with the sugar efflux site of the transporter (37, 33).

Fig. 2 shows the results of two representative experiments in which human and pigeon erythrocyte ghost GLUT was photolabeled using either [125I]ATB-BMPA or [125I]IAPS-forskolin. Although human erythrocyte GLUT1 was photolabeled in a 3-O-methylglucose- and cytochalasin B-inhibitable fashion, pigeon red cell GLUT was not detectable by these criteria. Moreover, total labeling of pigeon membrane proteins was not significantly changed following treatment of cells with FCCP.

We also measured equilibrium cytochalasin B binding to pigeon erythrocytes and erythrocyte ghosts. Again, we were unable to detect specific labeling that was inhibited by 3-O-methylglucose or by d-glucose ($n = 3$), nor was cytochalasin B binding affected by prior treatment of cells with metabolic poisons ($n = 4$). Cytochalasin B binding to pigeon erythrocytes is consistent with the presence of a single population of binding sites of $K_{\text{d(app)}} = 1 \mu\text{M}$ and density of $1 \times 10^6$ sites/cell (Fig. 2C). These experiments suggest that the failure to detect sugar-inhibitable ligand binding to pigeon red cell GLUT results from the low copy number of transporters present in these cells (approximately 100/cell based upon transport rates, see above). Fig. 2C demonstrates that more than 10,000 copies of GLUT/avian red cell must be present in order to detect these sites by the procedures employed in this study.

**Immunologic Approach**—Western analysis of pigeon red cell total proteins, ghost proteins, and integral membrane proteins (Fig. 3) demonstrates the presence of a low copy number (relative to human erythrocyte ghosts) of protein that cross-reacts with anti-GLUT1 COOH-terminal antibodies (C-IgGs). This species is characterized by a somewhat greater electrophoretic mobility than human red cell GLUT1 but migrates with proteins from rat and pigeon brain that cross-react with C-IgGs. Similar studies using anti-rat skeletal muscle GLUT4 COOH-terminal antibodies fail to detect immunologically related species in pigeon red cells but do detect related species in pigeon leg muscle (Fig. 3). Anti-human GLUT3 COOH-terminal antibodies fail to detect immunoreactive peptides in pigeon red cell and brain extracts but do cross-react with proteins in pigeon muscle (Fig. 3). These findings demonstrate that GLUT4 is effectively absent in pigeon red cells but that species immunologically related to

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**Table I**

<table>
<thead>
<tr>
<th>Physical properties of erythrocytes and 3-O-methylglucose transport characteristics of erythrocytes at 37°C</th>
<th>Human</th>
<th>Pigeon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell water content (fl)</td>
<td>66</td>
<td>88</td>
</tr>
<tr>
<td>GLUT1 (cell)$^{-1}$</td>
<td>300,000</td>
<td>196 ± 24</td>
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<tr>
<td>GLUT1 (mol-litter cell)$^{-1}$</td>
<td>7.8 × 10$^{-8}$</td>
<td>3.7 × 10$^{-9}$</td>
</tr>
<tr>
<td>$k_0$ (min)$^{-1}$</td>
<td>(2.3 ± 0.1) × 10$^{-3}$</td>
<td>(2.4 ± 0.3) × 10$^{-2}$</td>
</tr>
<tr>
<td>Poisoned</td>
<td>N/A</td>
<td>(2.6 ± 0.3) × 10$^{-3}$</td>
</tr>
<tr>
<td>$V_{\text{max}}$ net entry (mol-litter cell)$^{-1}$·H$_2$O$^{-1}$·min$^{-1}$</td>
<td>1.1</td>
<td>(6.4 ± 3.5) × 10$^{-4}$</td>
</tr>
<tr>
<td>Poisoned</td>
<td>N/A</td>
<td>(114.7 ± 23.2) × 10$^{-3}$</td>
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<tr>
<td>$K_{\text{m(app)}}$ net entry (mM)</td>
<td>3.8 ± 0.2</td>
<td>0.36 ± 0.02</td>
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<tr>
<td>Poisoned</td>
<td>N/A</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>$k_{\text{cat(GLUT1) (min)}^{-1}}$</td>
<td>145,000</td>
<td>1,730</td>
</tr>
<tr>
<td>Poisoned</td>
<td>N/A</td>
<td>30,980</td>
</tr>
</tbody>
</table>

*The equilibrium 3-O-methylglucose space (femtoliters) of human and pigeon erythrocytes |
*The concentration of GLUT1 proteins per human erythrocyte was taken from (30).
*The concentration of GLUT1 was computed as number of GLUT1/cell/cell water volume/Avgadron’s constant.
*The rate constant for leakage of sugar across the human erythrocyte membrane was taken from Ref. 31.
*N/A, not available.
* $V_{\text{max}}$ is computed as $V_{\text{max}}$/[GLUT1]. Human erythrocyte $V_{\text{max}}$ data are taken from Ref. 5.
GLUT1 are present in pigeon brain and erythrocytes. Assuming that the intensity of staining of GLUT1 in each lane of the autoradiograms of Fig. 3 is directly related to [GLUT1],

we calculate that pigeon red cells contain approximately 300 copies of GLUT1/erythrocyte.

The number of C-IgG-reactive sites present in leaky pigeon red cell ghosts was also determined by competition ELISA (Fig. 4). The utility of this assay is that it measures the ability of cells or purified GLUT1 to deplete medium of IgGs that react with purified GLUT1. Leaky pigeon erythrocyte ghosts deplete medium of GLUT1-reactive C-IgGs. Human erythrocyte leaky ghosts are 1.5 × 10³-fold more effective than pigeon ghosts in this regard. This suggests that pigeon erythrocyte ghosts expose 200 reactive sites to GLUT1-reactive C-IgGs. Similar experiments were also performed using anti-tetramer GLUT1 antibodies (δ-IgGs). These antibodies are raised against the native form of human erythrocyte GLUT1 (32) and react quantitatively with extracellular epitopes of GLUT1 (24). Again, intact human erythrocytes are 1.5 × 10³-fold more effective than intact pigeon red cells in their ability to deplete medium of tetrameric GLUT1-reactive δ-IgG. This suggests that pigeon red cells expose 200 copies of GLUT1 at the cell surface. Table II summarizes the results of 10 similar experiments.

δ-IgG binding to pigeon red cells is unaffected by prior treatment of cells with metabolic poisons (Figs. 4 and 5) nor does it affect 3-O-methylglucose uptake by control and poisoned cells (Table III). These observations indicate that δ-IgGs do not induce recruitment of GLUT1 to the cell surface. They also suggest that pigeon erythrocyte GLUT1 is quantitatively expressed at the plasma membrane at all times and is largely present as a GLUT1 tetramer.

Fig. 6 summarizes three separate experiments in which pigeon erythrocyte integral membrane proteins were subjected
to immunoprecipitation protocols prior to reconstitution using either preimmune rabbit serum or anti-GLUT1 antisera. These results demonstrate that anti-GLUT1 antisera quantitatively and specifically immunoprecipitate reconstitutable D-glucose transport activity from cholic acid extracts of pigeon red cell ghosts. This observation suggests that GLUT1 is the sole GLUT present in pigeon erythrocytes.

**DISCUSSION**

Sugar Transport in Control Erythrocytes—In most experiments we observed that cytochalasin B-inhibitable 3-O-methylglucose uptake by pigeon erythrocytes was either absent or occurred at very low rates. These results are consistent with those of Simons (15-17) and support the view that protein-mediated sugar transport is nominally absent in freshly drawn cells.

3-O-Methylglucose can penetrate control erythrocytes but does so largely via a cytochalasin B-insensitive pathway. At 5 mM sugar, the magnitude of this flux approaches 15 μmol/liter of cell water/min. This component of sugar uptake most likely results from nonspecific leakage (diffusion) of sugar across the cell membrane. Uptake is nonsaturable over the [3-O-methylglucose] range 0.1-10 mM and is unaffected by cytochalasin B or forskolin, two inhibitors of protein-mediated sugar transport (38-40). The permeability coefficient for 3-O-methylglucose “transbilayer diffusion” at 37 °C is approximately 1 × 10⁻⁹ cm s⁻¹, a value close to that for transbilayer diffusion in rat erythrocytes at 24 °C (3 × 10⁻¹⁰ cm s⁻¹ (31)).

Sugar Transport in Poisoned Cells—3-O-Methylglucose uptake by pigeon erythrocytes is stimulated during exposure to FCCP or cyanide (see here and Refs. 12, 15, and 16). 3-O-Methylglucose penetrates poisoned cells by two functional pathways. These consist of the leakage pathway (described above) plus a stereoselective saturable pathway that is inhibited by cytochalasin B and by forskolin. The rate of leakage is unaffected by metabolic poisons, whereas the rate of protein-mediated sugar uptake increases exponentially with duration of exposure to mitochondrial inhibitors (k = 0.018 min⁻¹; t₁/₂ = 40 min at 37 °C). Following 2 h of exposure to FCCP, the rate of protein-mediated 3-O-methylglucose uptake from medium containing 5 mM sugar approaches 100 μmol/liter of cell water/min, resulting in an approximate 8-fold increase in net sugar uptake over control cells. At physiologic D-glucose levels, intracellular D-glucose is virtually absent in both control and poisoned cells, although the rate of glucose uptake both approaches that of 3-O-methylglucose and is stimulated during poisoning (12, 14). These observations indicate that the rate of D-glucose utilization by avian erythrocytes exceeds the rate of uptake in both control and poisoned/hypoxic cells.

These findings strongly suggest that metabolic depletion results in derepression of inactivated or repressed protein-mediated sugar transport. Vₘₕₑₓ for protein-mediated 3-O-methylglucose uptake by pigeon erythrocytes is approximately 9,000-fold lower than that for 3-O-methylglucose uptake by human erythrocytes at the same temperature. Assuming the catalytic turnover numbers of human and pigeon erythrocyte sugar transporters are identical, this suggests that the pigeon erythrocyte contains approximately 30 copies of transport protein.

Ligand binding and photoaffinity labeling studies using cytochalasin B, forskolin, or ATB-BMPA failed to detect significant numbers of glucose transport proteins in pigeon erythrocytes but were successful in quantitating human erythrocyte transporter content. This failure does not result from the absence of cytochalasin B- or forskolin-binding sugar
and Ref. 16). The explanation must lie in the low transporter content of the pigeon red cell. Based upon the level of potent inhibitors of sugar transport in this tissue (see here plus 4 pg/ml FCCP (FCCP-treated cells) at 37 °C for 2 h. Cells were then incubated in saline (no addition) or in saline + δ-IgGs (50 nM serum/1 × 10⁶ cells) for an additional 2 h (plus δ-IgGs). Radiolabeled sugar was then added and uptake (at 37 °C) was measured at 0, 10, 20 and 60 min. The rate constant for uptake (k) was computed by nonlinear regression analysis assuming binding is characterized by simple saturation kinetics. The computed fits are as follows: control, maximum binding (B_max = (42.7 ± 1.2) × 10⁶ cpm, K_d = 0.87 ± 0.09 μl of δ-IgG; FCCP-treated cells, B_max = (51.7 ± 2.8) × 10⁶ cpm, K_d = 1.15 ± 0.15 μl of δ-IgG.

**TABLE II**

Quantitation of cellular GLUT1 content (copies per cell) by competition ELISA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>δ-IgGs</th>
<th>C-IgGs</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Red cell</td>
<td>178,090 ± 43,451</td>
<td>161,129 ± 57,812</td>
<td>156,103 ± 14,747</td>
</tr>
<tr>
<td>Leaky ghost</td>
<td>198,927 ± 29,538</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigeon red cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>147 ± 59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCCP</td>
<td>122 ± 60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaky ghost</td>
<td>205 ± 34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Three different antisera were used in separate experiments, δ-IgGs react with an extracellular epitope of GLUT1, C-IgGs react with an intracellular epitope only, whereas C2-IgGs react with both intra- and extracellular GLUT1 epitopes.

**FIG. 5.** Effects of FCCP poisoning on δ-IgG binding to pigeon erythrocytes. Erythrocytes were incubated in saline (C) or saline + 4 μg FCCP/ml (●) for 2 h at 37 °C. At this time varying concentrations of preimmune or δ-IgG were added (abscissa) and the suspensions incubated for an additional 2 h at 24 °C. The cells were washed extensively then incubated with [125I]-protein A for 2 h at 24 °C. The cells were washed, and cell surface [125I]-protein A was removed by a single wash in saline at pH 2.4 and then counted. The amount of δ-IgG-specific [125I]-protein A binding (counts/min/20 μl of packed cells) is shown on the ordinate. Curves were computed by nonlinear regression analysis assuming binding is characterized by simple saturation kinetics. The computed fits are as follows: control, maximum binding (B_max = (42.7 ± 1.2) × 10⁶ cpm, K_d = 0.87 ± 0.09 μl of δ-IgG; FCCP-treated cells, B_max = (51.7 ± 2.8) × 10⁶ cpm, K_d = 1.15 ± 0.15 μl of δ-IgG.

**FIG. 6.** Reconstitution of pigeon erythrocyte sugar transport function in proteoliposomes by cholate dialysis. Cholate-solubilized pigeon erythrocyte membrane proteins and lipids were reconstituted into egg PC proteoliposomes following immunodepletion using either preimmune (C, ●) or anti-GLUT1 (Δ, △) antisera. ([14C]3-O-Methylglucose uptake by proteoliposomes was measured in the absence (closed symbols: ●, △) or presence (open symbols: C, Δ) of 25 μM cytochalasin B. Each measurement was made in duplicate and was repeated on three separate occasions. The results are shown as mean ± 1 S.D. of these determinations.

**TABLE III**

Effects of δ-IgGs on 3-O-methylglucose uptake by pigeon red cells

<table>
<thead>
<tr>
<th>Condition*</th>
<th>3-O-Methylglucose uptake (k)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
</tr>
<tr>
<td>Plus δ-IgGs</td>
<td></td>
</tr>
<tr>
<td>Control Cells</td>
<td>0.00761 ± 0.00052</td>
</tr>
<tr>
<td>- CCB</td>
<td>0.00649 ± 0.00015</td>
</tr>
<tr>
<td>+ CCB</td>
<td>0.04510 ± 0.00206</td>
</tr>
<tr>
<td>FCCP-treated cells</td>
<td>0.00746 ± 0.00020</td>
</tr>
</tbody>
</table>

*3-0-Methylglucose (3 mM) uptake was measured in the presence (+CCB) or absence (−CCB) of 50 μM cytochalasin B.
† Cells were incubated in either saline (control cells) or in saline plus 4 μg/ml FCCP (FCCP-treated cells) at 37 °C for 2 h. Cells were then incubated in saline (no addition) or in saline + δ-IgGs (50 nM serum/1 × 10⁶ cells) for an additional 2 h (plus δ-IgGs). Radiolabeled sugar was then added and uptake (at 37 °C) was measured at 0, 10, 20 and 60 min. The rate constant for uptake (k) was computed as was done in Fig. 1A. Results are shown as computed best fit (±S.E.) of the nonlinear regression analysis.

transporters in pigeon erythrocytes, since these reagents are potent inhibitors of sugar transport in this tissue (see here and Ref. 16). The explanation must lie in the low transporter content of the pigeon red cell. Based upon the level of 3-O-
δ-IgGs and C2-IgGs. C1-IgGs react only with an intracellular epitope of human erythrocyte GLUT1, δ-IgGs bind only to extracellular epitopes of human erythrocyte GLUT1, whereas C2-IgGs react with both intra- and extracellular epitopes of GLUT1 (24, 28, 32). Similar reactivities were observed for these IgGs in pigeon red cells. As with human red cells (32), the number of C1G- and δ-IgG-reactive epitopes per pigeon red cell are indistinguishable. Since δ-IgG binding to pigeon red cells is without effect on both control and poisoned pigeon red cell 3-O-methylglucose uptake, and δ-IgG binding is unchanged in poisoned cells, these observations demonstrate the following. 1) δ-IgGs do not recruit GLUT1 to the plasma membrane. 2) Cell surface GLUT1 content is unaffected during transport stimulation. 3) Cell surface GLUT1 is largely tetrameric. 4) Cell surface GLUT1 accounts for total cellular GLUT1 content.

Our data demonstrate that GLUT1 is the only glucose transporter present in pigeon red cells. Quantitative immunoprecipitation of membrane GLUT1 using anti-GLUT1 IgGs results in the loss of reconstitutable sugar transport activity. Co-immunoprecipitation of other glucose transporter IgGs results in the loss of reconstitutable sugar transport activity. 36, 41, 42). In addition, pigeon brain extracts contain only GLUT1 (43-45), this result is not surprising.

Implications for Insulin-independent Sugar Transport Regulation—Sugar transport in avian erythrocytes is poised for regulation. Sugar metabolism is rate-limited by sugar uptake under normoxic conditions, because protein-mediated sugar transport is virtually absent. Inhibition of oxidative phosphorylation by cyanide or anoxia or the uncoupling of oxidative phosphorylation by FCCP triggers the functional expression of protein-mediated glucose transport, resulting in increased sugar uptake and glucose utilization. This response is also typical of mammalian cardiac and skeletal muscle (1).

The results we report here demonstrate that sugar transport is stimulated by an increase in the catalytic efficiency of cell surface GLUT1. Recruitment of GLUT1 or other glucose carrier isoforms to the plasma membrane of other cells is not observed in avian erythrocytes. Our results also suggest that plasma membrane GLUT1 function is repressed under normal conditions. Metabolic inhibition results in derepression of GLUT1, resulting in a catalytic efficiency that approaches that of GLUT1 in human erythrocytes. The factors that mediate GLUT1 repression under normoxic conditions are unknown at this time. Adipocyte sugar transport, unlike that in mammalian muscle and avian erythrocytes, is insensitive to metabolic depletion (3). This suggests that the pathway(s) that mediate GLUT1 repression is absent in adipose tissue. Protein-mediated transport rates and cellular [ATP] are inversely related in pigeon and goose erythrocytes (13, 17). ATP is known to be an allosteric inhibitor of GLUT1 function in human erythrocytes, whereas AMP and ADP serve as competitive inhibitors of ATP inhibition of transport (35, 46-50).

These considerations suggest a common theme for regulation of glycolysis and GLUT1-mediated sugar transport, namely allosteric inhibition by ATP at committed steps (transport, phosphofructokinase, and pyruvate kinase). Future studies must address this possibility and whether GLUT1 ‘‘repression’’ in the metabolically sensitive avian erythrocyte is related to ‘‘suppression’’ of GLUT1 and GLUT4 catalytic activities in the insulin-sensitive 3T3-L1 adipocyte (51-53).

Acknowledgments—We thank Dr. G. D. Holman for the generous gift of [3H](2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D- mannose-4-ylxyl)-2-ylaminomethyl. We are also grateful to Dr. M. F. Shanahan for the generous gift of 3-[3H]Ibido-4-azidophenethylamido-7-O-succinimidoyl-forskolin. Adipocyte low density lipoprotein fractions were kindly provided by Dr. M. P. Czech.

REFERENCES

A. Hansen and D. L. Diamond, unpublished observations.