Evidence for Interindividual Heterogeneity in the Glucose Gradient Across the Human Red Blood Cell Membrane and Its Relationship to Hemoglobin Glycation

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OBJECTIVE—To determine whether interindividual heterogeneity in the erythrocyte (red blood cell [RBC]) transmembrane glucose gradient might explain discordances between A1C and glycemic control based on measured fructosamine.

RESEARCH DESIGN AND METHODS—We modeled the relationship between plasma glucose and RBC glucose as the concentration distribution (C_i-to-C_o ratio) of a nonmetabolizable glucose analog ^{14}C-3-O-methyl glucose (^{14}C-3OMG) inside (C_i) and outside (C_o) RBCs in vitro. We examined the relationship between that distribution and the degree of glycation of hemoglobin in comparison with glycation of serum proteins (fructosamine), the glycation gap. A1C, fructosamine, and in vitro determination of the ^{14}C-3OMG distribution in glucose-depleted RBCs were measured in 26 fasted subjects.

RESULTS—The C_i-to-C_o ratio 0.89 ± 0.07 for 3-O-methyl-D-glucopyranose (3OMG) ranged widely (0.72–1.04, $n = 26$). In contrast, urea C_i-to-C_o (1.015 ± 0.022 [range 0.98–1.07], $P < 0.0001$) did not. Concerning mechanism, in a representative subset of subjects, the C_i-to-C_o ratio was retained in RBC ghosts, was not dependent on ATP or external cations, and was reestablished after reversal of the glucose gradient. The 3OMG C_i-to-C_o ratio was not correlated with serum fructosamine, suggesting that it was independent of mean plasma glucose. However, C_i-to-C_o did correlate with A1C ($R^2 = 0.19$) and with the glycation gap ($R^2 = 0.20$), consistent with a model in which differences in internal glucose concentration at a given mean plasma glucose contribute to differences in A1C for given level of glycemic control.

CONCLUSIONS—The data demonstrate interindividual heterogeneity in glucose gradients across RBC membranes that may affect hemoglobin glycation and have implications for diabetes complications risk and risk assessment. *Diabetes* **57:2445–2452, 2008**

A1C is the current gold standard for determination of chronic glycemic control in people with diabetes. Yet it is common to find hematologically normal people with diabetes in whom A1C appears discordant from other measures of glycemic control. Some have suggested the notion of a "hemoglobin glycation index" to assess A1C discordance from mean blood glucose (1–3). We have quantitated this discordance with the use of the glycation gap (GG), a measure of the disparity between two integrated measures of glycemic control, one intracellular in red blood cells (RBCs), A1C, and the other extracellular, glycated serum protein (GSP) measured as fructosamine (4). A non-zero GG could result from differences between the ambient glucose concentrations or rates of glycation in the intracellular and extracellular compartments, and/or interindividual differences in the turnover/metabolism of underlying proteins. We have demonstrated that the GG is reproducible within subjects over time and is associated with important clinical end points. In longstanding type 1 diabetes, there is a over a twofold rise in prevalence of nephropathy in patients with high GG versus low GG (4). GG is also a predictor of retinopathy (5). Furthermore, approximately one-third of the heritable component of A1C (6) is shared in common with a heritable component of the GG (7), suggesting a genetically determined mechanism for discordance between fructosamine and A1C.

As indicated by Higgins et al. (8), two factors other than plasma glucose influence the level of glycated hemoglobin: lifespan and "glucose permeability" of the RBC. Yet there has been little systematic investigation of how interindividual variability in these parameters may affect the relationship between measured A1C levels and plasma glucose. In another study, we examined differences in RBC survival and reported variability that can produce significant deviations of A1C from expected values (9). We focus here on glucose permeability, i.e., the concentration of glucose within versus outside the RBC. Glucose transport across the RBC membrane mediated by the GLUT1 transporter is complete within seconds at body temperature (10). Thus, in addition to plasma glucose and RBC lifespan, "equilibrium" intracellular glucose, rather than the rate of glucose transport, is likely the determining factor in hemoglobin glycation.

Over 80 years ago, Somogyi (11) found that the "corpuscular sugar–to–serum sugar" ratio averaged 0.77. Although it varied among healthy subjects between 0.66 and 0.95, the ratio did not change with glucose challenge and was similar between a diabetic and nondiabetic subject. In the Higgins et al. (8) description of the variation in A1C

between animal species in relation to glucose permeability of the RBC, the erythrocyte glucose–to–plasma glucose concentration ratio was 0.67 ± 0.14, demonstrating greater variation than expected for experimental technique or RBC water space, which is very tightly regulated (mean corpuscular hemoglobin concentration [MCHC] range 32–35%) (12). This suggests the possibility of substantial interindividual variation in erythrocyte glucose. Similarly, Gould and Yudkin and coworkers (2,3) reported systematic variation in the relationship between A1C and fasting plasma glucose in a nondiabetic population that correlated with erythrocyte glucose. A significant difference in erythrocyte glucose–to–plasma glucose concentration ratio was found under non–steady state conditions: 0.74 ± 0.12 in "low glycators" and 0.98 ± 0.06 in "high glycators" ($P < 0.01$) (3) after accounting for cell water (B. Gould, personal communication). The similarity among the results of Somogyi, Higgins et al., and Gould et al. are sufficient to propose that there are individual differences in how sugar distributes into the RBC. Yet, one of the key assumptions in the assessment of glycemic control by A1C is that glucose in the intra-erythrocyte space (which determines hemoglobin glycation) bears a common relationship to glucose in the plasma space (which defines glycemic control). A systematic difference in glucose permeability would constitute a source of intersubject differences in A1C at a given average plasma glucose concentration.

In this study, we sought to test the hypothesis that interindividual heterogeneity of the intracellular-to-extracellular glucose ratio contributes to variability in the relationship of A1C to fructosamine. We confirmed the existence of a glucose gradient across the human RBC membrane by measuring the distribution of the nonmetabolizable glucose analog, ^{14}C-3-O-methyl glucose (^{14}C-3OMG) into the RBC, which eliminated the potential contribution of metabolism of glucose to such measurements. We validated our methodologies by demonstrating the expected equilibration of another small molecule, urea, and eliminated intracellular hemoglobin, ATP, and transmembrane cation gradients as contributors to the glucose gradient. Finally, we demonstrate that the variation of the transmembrane glucose gradient among individuals correlates with the glycation gap and A1C but not with fructosamine.

RESEARCH DESIGN AND METHODS

3-O-methyl-D-glucopyranose (3OMG), 3-O-(^{14}C-methyl)-D-glucose, mercuric chloride, phloretin, cytochalasin B, Drabkin's reagent, and hemoglobin standards were obtained from Sigma Chemical (St. Louis, MO). PBS, perchloric acid, Scintisafe Plus cocktail, and STANBIO cyanmethemoglobin standards were from Fisher Scientific (Pittsburgh, PA).

Subjects recruited were ≥ 14 years of age (Table 1). Exclusion criteria included baseline serum creatinine >1.5 mg/dl, urine albumin >200 μg/min (timed collection) or >179 μg/mg creatinine (spot collection), transaminases more than three times the upper limit of normal, New York Heart Association heart failure stage ≥ 3, hematocrit <34%, reticulocyte count >2%, evidence of hemoglobinopathy on high-performance liquid chromatography (HPLC) analysis of hemoglobin, active infection, or an underlying illness known to be associated with body wasting (e.g., malignancies or tuberculosis). All research procedures were approved by the University of Cincinnati Institutional Review Board.

Preparation of erythrocytes and ghosts. Blood was drawn in heparin-coated tubes and processed as previously described (13) with modifications from Klepper et al. (14). Blood specimens were centrifuged at 2,500 rpm for 5 min to sediment RBCs. Serum and buffy coat were aspirated. To minimize differences in cellular sugar transport experiments resulting from diabetic subjects' plasma glucose in vivo, erythrocytes were incubated at 37°C twice for 30 min in a 10-fold excess of PBS, then washed twice in PBS at ice temperature. Assays were standardized by measurement of hemoglobin

TABLE 1
Study subject characteristics

Age (years)	44 ± 11
N	26
Sex	
Female	13
Male	13
Glucose tolerance status and diabetes treatment	
Nondiabetic	5
Type 1 diabetic	10 (all insulin only)
Type 2 diabetic	11 (6 insulin, 7 metformin, 2 sulfonylurea, 4 thiazolidinediones)
Race (n)	
Caucasian	20
African American	5
South Asian	1
Glycemic control	
Fasting plasma glucose (mg/dl)	168 ± 76 (56–309)
A1C (%)	7.6 ± 2.1 (4.6–12.4)
Fructosamine (μmol/l)	322 ± 82 (191–505)

Data are means $\pm$ SD and means $\pm$ SD (range) unless otherwise indicated.

spectrophotometrically with Drabkin's or Stanbio reagents and adjusting all erythrocyte suspensions to hemoglobin 15 g/dl for 3OMG transport. To prepare pink ghosts, packed RBCs were lysed in 10 volumes of 5 mmol/l phosphate buffer, pH 7.5. For white ghosts, erythrocytes were lysed in 50 V of the hypotonic buffer. Ghosts were centrifuged; pink ghosts were washed twice with cold PBS, and white ghosts were washed with lysis buffer until the supernatant was clear. For the depletion of intracellular ATP, 8 units/ml of the enzyme apyrase was included in the lysis buffer. Ghosts were resealed with 150 mmol/l KCl in lysis buffer at 37°C for 1 h. Resealed ghosts were sedimented, the supernatant was aspirated, and the ghosts were decanted (to remove the nonlysed cell button adhering to the bottom of the centrifuge tube) and subjected to an additional wash/centrifugation cycle. The resulting cells are impermeable to ^{14}C-maltose (the 3OMG-to-maltose space ratio of RBC ghosts is 100:1) (15).

Erythrocyte membrane glucose gradient in vitro methods. Erythrocyte membrane glucose gradient in vitro methods were developed from those used for zero-*trans* glucose influx kinetics (10,14,16,17). 3OMG, which undergoes phosphorylation several orders of magnitude more slowly than glucose and is virtually nonmetabolizable (18–20), has been used extensively in glucose transport studies (10). Aliquots of erythrocytes (100 μl), prepared as above, were suspended in 200 μl ^{14}C-labeled (0.5 μCi/ml final) and unlabeled 3OMG, yielding a final concentration of 10 mmol/l for single C_i-to-C_o ratio determinations, or 0.3–20 mmol/l to measure C_i-to-C_o ratio versus 3OMG. Incubations were stopped by the addition of ice-cold stop solution consisting of 50 μmol/l phloretin and 100 μmol/l mercuric chloride in PBS at various time points. Zero time values were obtained by the addition of stop solution to the RBC suspension before addition of ^{14}C-3OMG. Cells were centrifuged at 14,000 rpm for 30 s, and 100-μl aliquots of the supernatant were removed to sample extracellular counts. The RBC pellet was washed twice with stop solution on ice and then lysed with 0.5 ml 3% perchloric acid. After centrifugation at 14,000 rpm for 10 min at room temperature, 200-μl aliquots of the perchloric acid extract were collected to sample intracellular counts. After scintillation counting, the concentration of ^{14}C-3OMG (in counts per minute per milliliter) outside the cell (C_o) and inside the cell (C_i) was then calculated. C_i was determined as counts per minute per milliliter cell water. Assuming identical internal- and external-specific activities, the ratio counts of C_i to C_o would then be equivalent to the ratio of inside to outside concentrations of unlabeled compound.

An alternative indirect method was developed for comparison of C_i to C_o for ^{14}C-3OMG and ^{14}C-urea (final unlabeled urea, 2 mmol/l). Tracer was added to RBC suspensions adjusted to 15 g/dl hemoglobin (45% hematocrit) and incubated to steady state. After centrifugation, aliquots of supernatant and ^{14}C-tracer stock solution were counted for radioactivity. From the supernatant and stock solution counts, the hematocrit, and the cell water fraction estimated from MCHC, the concentration of counts in cell water C_i and the corresponding C_i-to-C_o ratio were calculated.

Results are reported as the mean of triplicate measurements. In representative experiments, there was no change in the hematocrit from the beginning

to the end of the incubation, despite mild visible evidence of hemolysis (<0.6% by absorbance; no difference in hemolysis between 3OMG and urea incubations).

Calculation of cell water. The cell water was calculated for each sample from the MCHC determined on a complete blood count sent simultaneously to the clinical laboratory according to the following equation (21):

$$(1 - CWF_{sample}) = (1 - CWF_{reference}) \times MCHC_{sample}/MCHC_{reference}$$

where cell water fraction $(CWF)_{reference}$ was measured as 0.669 (volume of solvent water per volume of cells) on a sample of normal cells with measured $MCHC_{reference} = 33.5$ g/dl. In preliminary experiments, CWF was determined experimentally on several blood samples and was comparable with that calculated by MCHC.

Analytic methods. A1C was measured using HPLC ion exchange (Tosoh, Tokyo, Japan) (interassay coefficient of variation [CV] 2.9% at A1C 5.5% and 1.9% at A1C 10.0%). Fructosamine was measured at Quest Diagnostics by autoanalyzer (Roche Diagnostics) with a nitroblue tetrazolium reaction, interassay CV 2% (4).

Statistical analysis. The GG was computed based on a reference regression line relating A1C and fructosamine as previously reported (4). All data are reported as means ± SD, unless otherwise indicated. We used Pearson's correlation coefficients and linear regression to evaluate relationships between variables. Analyses were conducted using SPSS version 15.0 (SPSS, Chicago, IL) and JMP (SAS Institute, Cary, NC).

RESULTS

Evidence for a glucose concentration gradient in human erythrocytes under conditions of steady state. Human erythrocytes incubated with a series of concentrations of 3OMG rapidly reached a steady state within 5 min at 37°C (Fig. 1A). This was stable well beyond the 60-min time point chosen for ongoing comparative analysis (data not shown). However, ^{14}C-3OMG inside (C_i) and outside (C_o) the cell did not equalize, and the C_i-to-C_o ratio at steady state at 37°C departed significantly from unity. C_i-to-C_o increased with increasing 3OMG (Fig. 1B), but the glucose gradient expressed as the difference between inside and outside was actually higher at higher concentrations. C_i-to-C_o measured at physiological concentrations of 10 mmol/l 3OMG was <1 in 25 of 26 subjects, with a broad distribution of values (0.89 ± 0.07) (Fig. 1C). For determinations of C_i-to-C_o on fresh samples assayed over a 12-day period, within subject CV ranged from 5 to 7%. There was no correlation of C_i-to-C_o with mean corpuscular volume (MCV) or MCHC.

Comparison of 3OMG and urea gradients. To test whether the transmembrane asymmetry would also be observed with a distinct small molecule thought to fill the RBC water space, parallel incubations were conducted simultaneously with ^{14}C-3OMG and ^{14}C-urea, each in quadruplicate (Fig. 2). Since rapid efflux of urea from RBC precluded washing of cells, C_i for both compounds in these studies was calculated from measured counts in the ^{14}C-labeled stock solution added to the suspension and in the supernatant representing the extracellular space (see RESEARCH DESIGN AND METHODS). A wide distribution and departure from unity of 3OMG C_i-to-C_o ratios is apparent, confirming the previous observation (Fig. 1C). In contrast, urea C_i-to-C_o was tightly clustered at 1.015 ± 0.022. The calculations of C_i for both 3OMG and urea used the same estimates of cell volume, MCHC, and cell water; therefore, the C_i-to-C_o ratio of unity for urea validates these estimates. In eight subjects, C_i-to-C_o ratios were assessed using both direct and indirect measures for ^{14}C-3OMG, along with simultaneous measurement of the ^{14}C-urea concentration ratio (indirect) on the same blood samples. The results were significantly lower ($P < 0.0001$ each; paired t test) for ^{14}C-3OMG with both the direct (0.93 ± 0.02) and indirect methods (0.86 ± 0.05) compared with
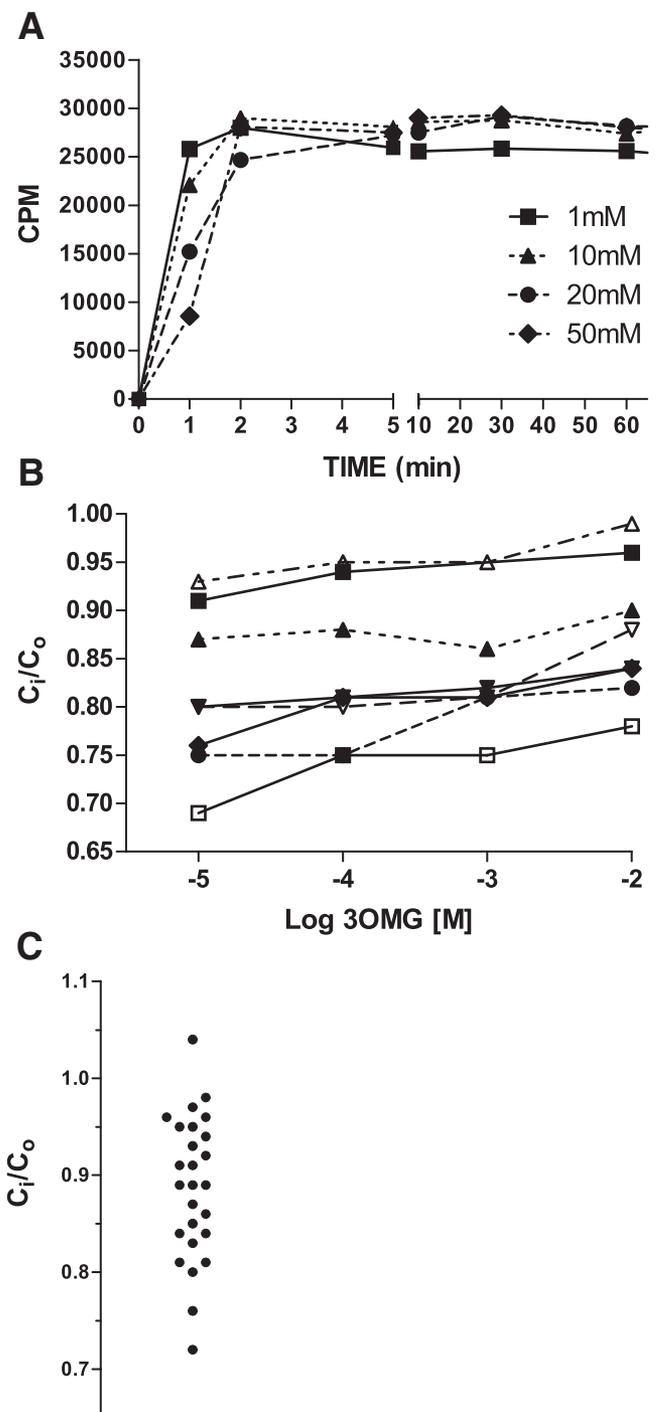
FIG. 1. *A*: Time course of ^{14}C-3OMG uptake into glucose-depleted human erythrocytes at 37°C at a series of initial external sugar concentrations. Values shown are mean of triplicate incubations. *B*: Dependence of the 3OMG gradient on external 3OMG. The concentration gradient decreases (i.e., C_i-to-C_o approaches 1) with increasing 3OMG across a concentration range broader than the physiologic ($n = 7$; different symbols and lines denote individual subjects). *C*: Distribution of C_i-to-C_o determined at steady state in human subjects (Table 1).

^{14}C-urea (1.04 ± 0.04). C_i-to-C_o ratios for ^{14}C-glucose, ^{14}C-galactose, and ^{14}C-2-deoxy-glucose were similar to those with ^{14}C-3OMG (data not shown), providing confirmation among a series of sugars transported by GLUT1. These results are consistent with previous findings that the "nonsolvent" water compartment in red cells is very small, i.e., all the water of RBCs is available as solute for small
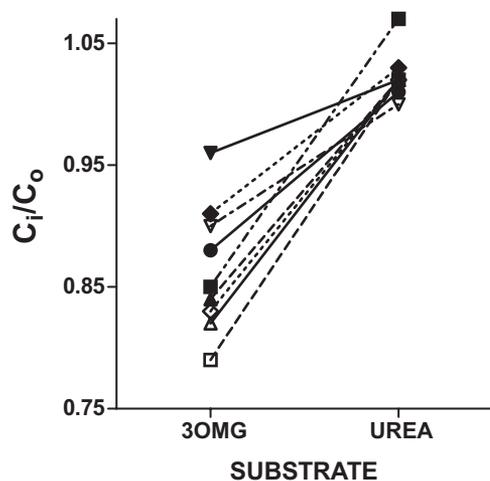
FIG. 2. Paired values of the C_i-to-C_o ratio determined for 3OMG vs. urea within subject are different ($P < 0.0001$). The C_i-to-C_o ratio approximates unity and has a narrow distribution for urea in all subjects but is below unity and more heterogeneous in the same population for 3OMG.

molecules such as urea. Bookchin et al. found similar results for glucose (22). Thus, C_i-to-C_o ratios <1 are not due to variations or error in estimation of water content of the cells.

Transmembrane gradient is maintained in RBC ghosts. Some studies in the literature suggest that the high concentration of hemoglobin may "compartmentalize" glucose, generating an apparent 3OMG gradient (23). To test this possibility, C_i-to-C_o determinations were conducted in "pink" and "white" ghosts of red cells. Pink ghosts represent the resealed product of lysing red cells in a 10-fold excess of 5 mmol/l sodium phosphate hypotonic buffer, reducing the MCHC from 33.3 to 1.6–2.6 g/dl; white ghosts result from conducting analogous lysis in a 50-fold excess of hypotonic solution, further reducing MCHC to 0.011–0.037 g/dl. These procedures raise the CWF from ~0.675 to 0.973 and 0.99, respectively. Preparation of each ghost form did not result in disappearance of the C_i-to-C_o gradient (both C_i and C_o measured directly using stop solution). In fact, the C_i-to-C_o gradient became more pronounced (Fig. 3A and B). This excludes hemoglobin as a source of the C_i-to-C_o gradient.

Effect of perturbing energy status on observed gradient. The C_i-to-C_o gradient was not dependent on the normal cellular Na/K gradient. Cells incubated in either isotonic NaCl or isotonic KCl had identical C_i-to-C_o gradients (Fig. 4A). The C_i-to-C_o gradient was also not dependent on ATP. Figure 4B demonstrates that C_i-to-C_o was the same in ghosts made with and without the ATP-destroying enzyme apyrase, which is present during resealing.

Testing for an "impurity" in the labeled 3OMG. Quality control analysis on the [14]C-3OMG provided by the manufacturer using HPLC indicated a homogeneous species. It could be hypothesized that another molecular species that fails to cross the red cell membrane, e.g., an enantiomer, might not be detected by that technique, giving rise to an artifactual 3OMG C_i/C_o gradient (24,25). We chose a functional approach to test for such an impurity in the tracer. If a nontransportable impurity were responsible for the C_i-to-C_o gradient, incubation with RBCs would result in uptake solely of transportable "pure" tracer. Incubation of those cells with fresh tracer-free medium would then elute the tracer from the cells and
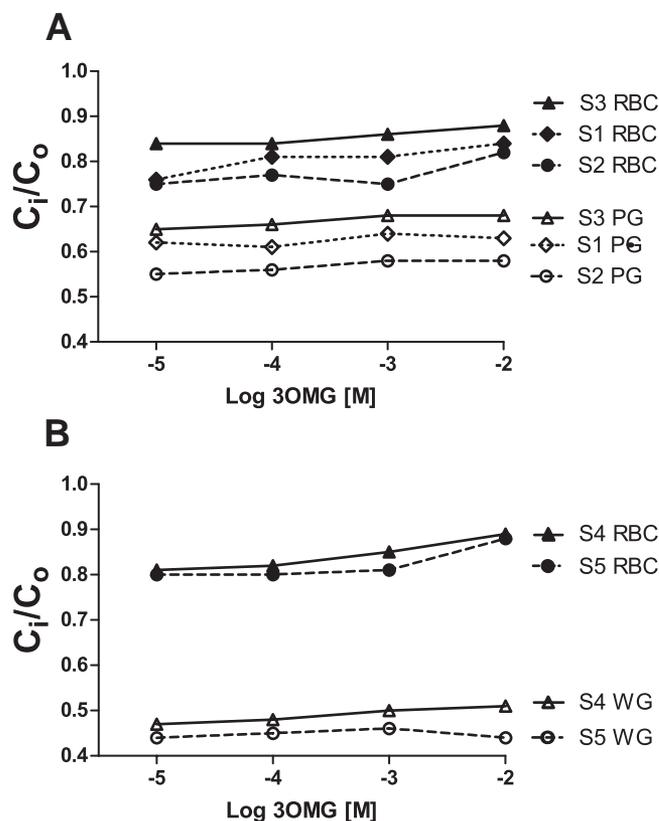
FIG. 3. 3OMG (3-O-methyl-glucose) C_i-to-C_o dependence on external 3OMG in pink (PG) (A) and white (WG) (B) erythrocyte ghosts compared with intact RBCs (S1–S5 denote different human subjects). The 3OMG concentration gradient was not reduced, and in fact was more pronounced as hemoglobin decreased. A is representative of 17 experiments in eight subjects, and B is representative of one experiment in each of three subjects.

result in an "impurity-depleted" eluate. Subsequent incubation of fresh RBCs with this impurity-depleted eluate should therefore eliminate the C_i-to-C_o gradient, if the impurity were the source of the apparent gradient. Therefore, a standard zero-*trans* experiment was performed with and without the following modification: the supernatant from the initial incubation for 1 h was removed by centrifugation and replaced with 200 μl fresh tracer-free medium for 60 min at 37°C. This medium (termed the "eluate") was then separated from the cells by centrifugation, and 200 μl was added to a fresh suspension of 100 μl RBCs in 10 mmol/l 3OMG. Similar C_i-to-C_o gradients were observed with medium containing fresh unmodified tracer (0.92 ± 0.01) and with the eluate (0.91 ± 0.04, $n = 11$, $P =$ NS) whose tracer had to have been able to enter and then exit from cells to which it had previously been exposed. This indicates that a nontransportable impurity is unlikely to account for the deviation of the C_i-to-C_o ratio from 1.0.

Effect of reversal of initial glucose gradient. The previous experiments represented influx of glucose into the cell down an outside-to-inside concentration gradient. We attempted to reverse this gradient by first equilibrating cells in [14]C-3OMG with 0.2 mmol/l 3OMG and then expanding the extracellular space with an equal volume of sugar-free buffer (which thereby dilutes the extracellular sugar concentration from 0.2 to 0.1 mmol/l) to examine the steady-state gradient achieved when flux is in the opposite direction. The lower concentration of 3OMG was used to allow for studying the effects of cytochalasin B, a well-documented inhibitor of GLUT1-mediated glucose trans-

A

B
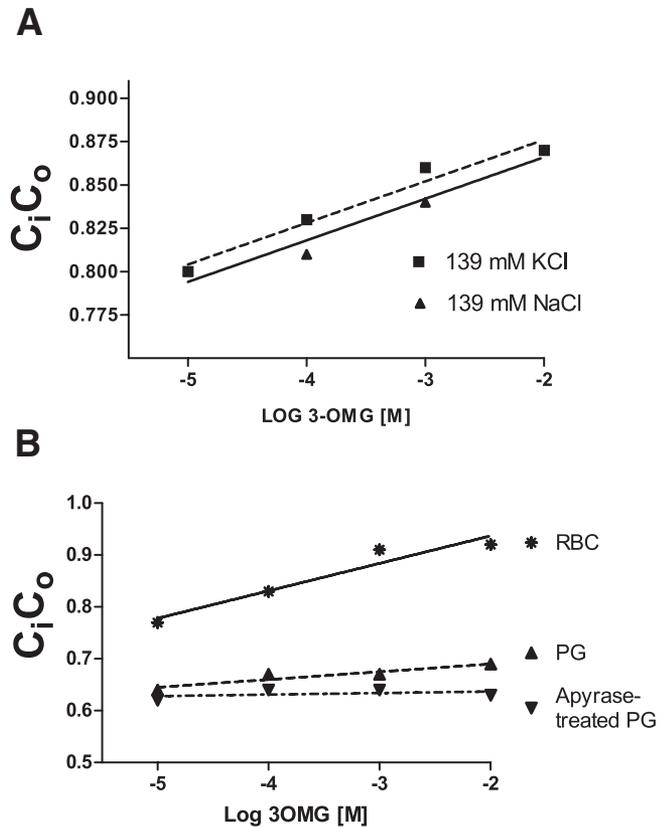
FIG. 4. *A*: **Effect of transmembrane cation gradient on the 3OMG C_i-to-C_o dependence on external 3OMG. The dependence of 3OMG C_i-to-C_o on 3OMG was compared in extracellular medium containing 137 mmol/l KCl vs. the standard PBS containing 139 mmol/l NaCl. The use of KCl buffer failed to overcome the gradient. The data are representative of one experiment from each of three subjects.** *B*: **ATP effects. Preparations of pink ghosts (PG) sealed with the addition of the enzyme apyrase to deplete intracellular ATP failed to shift C_i-to-C_o toward unity. However, the preparation of ghosts reduced the 3OMG dependence of the C_i-to-C_o ratio and was further reduced by apyrase. The data are representative of one experiment in each of four subjects.**

FIG. 5. **Membrane gradient reversal. In this experiment, RBCs were first incubated with 3OMG until an apparent steady-state outside-to-inside gradient was reached. Next, the samples (*arrow*) were diluted twofold to expand the extracellular volume. If the observed gradient was due to a failure to reach true equilibrium, then the gradient would be expected to reverse after dilution. However, after dilution, there was a prompt reestablishment of essentially the same steady-state outside-to-inside gradient, indicating that the sugar moved up a concentration gradient. The data are representative of one experiment in each of 15 subjects.**

port (26,27). Despite reversing the direction of the initial gradient, the final steady-state C_i-to-C_o ratio was not significantly altered (Fig. 5) and there was no effect of incubation with cytochalasin B (data not shown). This finding is striking, in that cellular glucose concentration falls below extracellular glucose concentration. We do not have a mechanistic explanation for this phenomenon, given the independence of C_i-to-C_o ratios from ATP and cation gradients. However, this finding suggests that the C_i-to-C_o gradient would persist in vivo, tracking changes in plasma glucose concentrations whether rising or falling (see DISCUSSION).

Relationship of A1C and GG with C_i-to-C_o ratio. C_i-to-C_o ratio, which varied among individuals, correlated positively and significantly with both A1C and GG (Fig. 6). Higher A1C levels were associated with higher C_i-to-C_o ratios. Likewise, individuals with higher C_i-to-C_o ratios had higher GG values, i.e., when the intra-erythrocyte concentration of sugar was higher relative to extracellular sugar (represented by higher C_i-to-C_o ratios), so was the glycation of the intracellular protein hemoglobin in comparison with the glycation of extracellular proteins (reflected in higher GG). This is consistent with variation in the C_i-to-C_o ratio among individuals contributing to A1C levels and to the discordance between A1C and glycemic control. In contrast, there was no correlation of either C_i-to-C_o or GG

with serum fructosamine (Table 2), indicating that C_i-to-C_o and GG were not influenced by glycemic control per se. In statistical terms, the GG is the residual of the regression of A1C on fructosamine. It should be randomly distributed with respect to fructosamine, hence, as observed, not correlated with fructosamine. On the other hand, GG would be expected to correlate with A1C because a high GG suggests that the A1C is higher than would be predicted and a low GG suggests that the measured A1C is lower than predicted from fructosamine. The correlation of C_i-to-C_o with GG is consistent with the GG being determined in part by the C_i-to-C_o ratio.

DISCUSSION

The key findings in this study are that *1*) at steady state, there is a sugar concentration gradient across the human erythrocyte membrane that varies between individuals; *2*) the gradient is not dependent on glucose metabolism, hemoglobin concentration, ATP, or the transmembrane cation gradient; *3*) the glucose gradient correlates with A1C but is not related to a non-RBC glycated protein measure of glycemic control, suggesting that it reflects an influence on A1C unrelated to plasma glucose. Taken together, these data are consistent with the hypothesis that variability in the intracellular (erythrocyte glucose) relative to extracellular glucose significantly contributes to interindividual variation in A1C. This has direct implications for the mechanisms determining A1C and its clinical interpretation as a measure of glycemic control and risk prediction.

Although use of a nonphysiologic sugar to exclude the effects of glucose metabolism and defined media are potential limitations to generalizability in vivo, results were similar with physiologic sugars. We eliminated a smaller distribution space for glucose in the RBC than the cell water volume estimated from the MCHC as a potential explanation of a steady-state transmembrane glucose gradient. Cell water volume estimated from MCHC and vali-
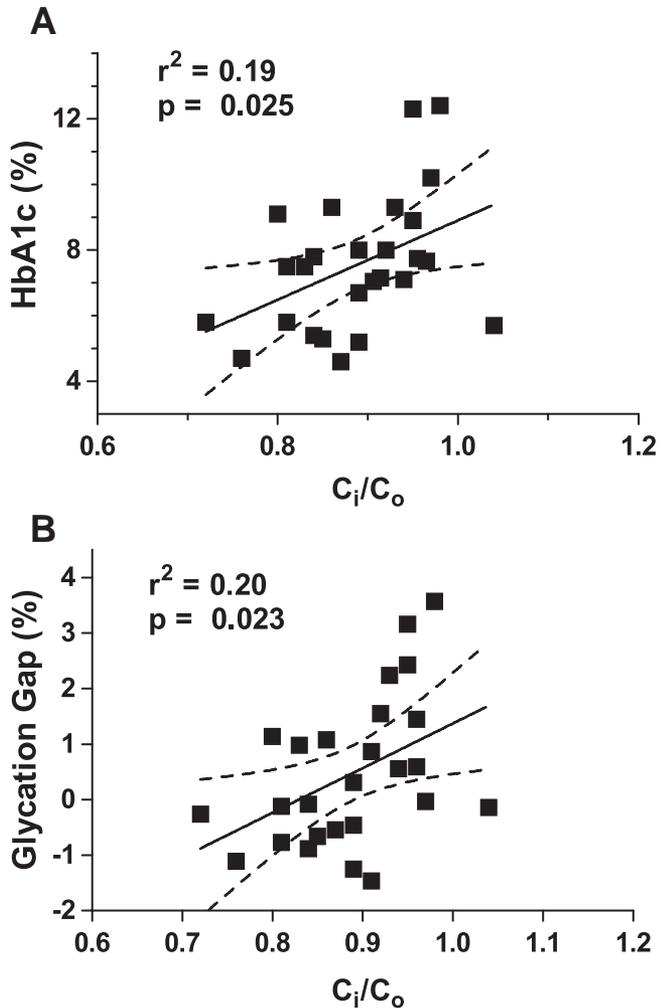
FIG. 6. A1C (*A*) and GG (*B*) rise as the ^{14}C-3OMG C_i-to-C_o ratio increases. The GG is a measure of variance in A1C relative to glycated serum proteins. Across the population, intracellular sugar rises relative to extracellular, as does hemoglobin glycation relative to the glycation of extracellular proteins. Values of r^2 are shown to demonstrate the fraction of the variance in A1C and GG accounted for by C_i-to-C_o ratio. In contrast (Table 2), fructosamine has no significant slope relative to C_i-to-C_o ratio.

dated by urea C_i-to-C_o = 1.0, is consistent with the long-standing observation that virtually all water in RBCs was available for solvation of small molecules (28,29). More recently, Bookchin et al. (22) showed that even the water contained in the highly structured dense protein domain of intracellular polymerized hemoglobin S was accessible to small molecules, including glucose. Furthermore, the C_i-to-C_o gradient did not depend on high intra-

TABLE 2
Correlation coefficients (*r*) and *P* values between A1C, fructosamine, GG, and C_i-to-C_o ratio*

	r	*P*
A1C vs. fructosamine	0.77	<0.001
A1C vs. GG	0.76	<0.001
GG vs. fructosamine	0.17	0.40
C_i-to-C_o vs. A1C*	0.44	0.025
C_i-to-C_o vs. GG	0.45	0.023
C_i-to-C_o vs. fructosamine	0.23	0.26

*Ratio of sugar concentration inside to outside measured as ^{14}C-3OMG CPM inside vs. outside (see Fig. 6 legend).

cellular hemoglobin concentrations. The presence of intracellular membrane vesicles could conceivably represent a compartment inaccessible to glucose. Such endocytic vesicles have been demonstrated in sickle RBCs but not in normal RBCs (30). Thus, there is no evidence in RBCs for a substantial water compartment inaccessible to glucose that could explain C_i-to-C_o ratios <1.

Another potential explanation of the failure of glucose concentrations to equilibrate across the membrane is that kinetic characteristics of the glucose transport system limits transport before equilibration occurs, i.e., glucose influx is shut down before equal concentrations are reached. However, the reestablishment of C_i-to-C_o when external glucose concentrations were diluted (Fig. 5) is inconsistent with this mechanism. This experiment in fact suggests a third possibility: a mechanism for net export of glucose from the cell. There has been no evidence to date for active transport of glucose in or out of the RBC, and the C_i-to-C_o gradient was not dependent on ATP or cation gradients that might serve as energy sources; there is not an obvious alternative candidate in the artificial media used for these 3OMG uptake experiments. Other molecules share the GLUT1 transporter (31,32), but competition is unlikely in the artificial media used, especially given the wide concentration range over which the sugar C_i-to-C_o gradient is preserved (Fig. 1*B*). Nevertheless, there are a number of aspects of the glucose transport system that suggest more complex characteristics and regulation than that of a simple "facilitated diffusion" mechanism. Red cell sugar transport displays kinetic asymmetry in which V_{max} and K_m for sugar exit are greater than V_{max} and K_m for sugar entry (16,17,33–35). This does not result in net glucose export when intracellular sugar = extracellular sugar because the ratio of V_{max} to K_m for net exit and entry are identical (36). Nevertheless, the transient fall in 3OMG concentration below external concentrations has been seen in rapid counter-flow studies, and the rapid 3OMG exit observed in human RBCs and ATP-containing RBC ghosts (A.C. and J.M. Leitch, unpublished observations) supports this alternative possibility that RBCs contain a second glucose transport system that actively exports sugars (37). The identity of the putative glucose exporter is not known (37), and the energy source driving glucose exit is unclear.

As has recently been indicated, the GG (or the analogous hemoglobin glycosylation index) (1,38–40) is not independent of A1C (41,42). It does however allow quantitation of sources of variation in A1C (43). In this construct, in which the GG is defined as the difference between measured and predicted A1C, the C_i-to-C_o ratio is linked not only to A1C but also with the fraction of the variance in A1C that is not shared in common with the integrated extracellular measure of glycemic control, fructosamine.

Whatever the molecular mechanism involved in establishing the glucose gradient of RBCs, the observed variation among individuals in C_i-to-C_o ratios from ~0.7 to 1.0, could lead to substantial differences in hemoglobin glycation for a given plasma glucose, with significant clinical implications. Critical clinical decisions are made based on A1C in the 6–8% range (44–48). The difference between one individual with a C_i-to-C_o of 0.75 and another with C_i-to-C_o of 0.99 could be an ~25% difference in the level of hemoglobin glycation for a given mean plasma glucose, which translates to a difference of 1.5–2.0 A1C percentage points. We speculate that the interindividual variation in

C_i-to-C_o may contribute to the observed genetic variation in the incidence of diabetes complications. It will be important to understand whether the phenomenon we are describing in RBCs extrapolates to other cell types, notably endothelial cells. If so, then the C_i-to-C_o glucose gradient may have implications not only for A1C, but also for those intracellular glucose concentrations that affect the rate of progression of diabetes complications themselves.

In conclusion, we have identified a variable in the RBC—the *trans*-membrane glucose gradient—that is a strong candidate to introduce interindividual variability into the rate of A1C formation. Recognizing this variable has important implications for clinical interpretation of this widely used test. Understanding the underlying mechanism may have fundamental implications for interindividual variation in the frequency of diabetes complications in relation to apparent glycemic control.

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