Red Cell Sorbitol

An Indicator of Diabetic Control

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SUMMARY

Intact human erythrocytes accumulate intracellular sorbitol in response to the medium's glucose concentration during in vitro incubations. Sorbitol was identified and measured both enzymatically and by gas-liquid chromatography. The sorbitol produced is most likely a result of the activity of aldose reductase, since (1) a low glucose concentration in the medium elicits this response, and (2) this activity is completely blocked by tetramethylene glutaric acid, a specific inhibitor of aldose reductase. Erythrocyte sorbitol levels in insulin-dependent diabetics are clearly above those of nondiabetics after an 8 h fast. A good correlation exists between red cell sorbitol content and coincident plasma glucose concentrations. Individual exceptions to this rule exist, however, and suggest that red cell sorbitol levels may provide information about in vivo polyol pathway activity that may be important in the pathogenesis of diabetes-associated complications. DIABETES 29:861–864, November 1980.

Sorbitol is an intermediate in the polyol pathway that converts glucose to fructose. Aldose reductase (alditol: NADP oxidoreductase, EC 1.1.1.21) converts glucose to sorbitol, and sorbitol dehydrogenase (L-iditol: NAD oxidoreductase, EC 1.1.1.14) converts sorbitol to fructose.

$$\text{D-glucose} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{Aldose Reductase}]{\text{The Polyol Pathway}} \text{Sorbitol} + \text{NADP}^+$$

$$\text{Sorbitol} + \text{NAD}^+ \xrightarrow[\text{Sorbitol Dehydrogenase}]{} \text{D-fructose} + \text{NADH} + \text{H}^+$$

The activity of this minor pathway for glucose metabolism is independent of insulin. As the ambient glucose concentration rises above physiologic levels (about 10 mM) the activity of the polyol pathway increases.[1] Sorbitol does not readily diffuse across cell membranes, and the intracellular accumulation of sorbitol has been reported to be one of the factors responsible for cataracts,[2] peripheral neuropathy,[3] and possibly the vascular complications[4] of diabetes mellitus.

Sorbitol has been measured in human red cells by Travis et al;[5] the results suggest that aldose reductase may be present in this tissue. Those data have been questioned, since sorbitol was measured with commercially available sorbitol dehydrogenase;[6] certain preparations of this enzyme may not be highly specific for sorbitol, but may also oxidize other polyols, such as mannitol, xylitol, or galactitol. The polyols of the human red cell were further characterized by gas-liquid chromatography of trimethylsilyl esters prepared from protein-free filtrates of the erythrocyte. A hexitol peak was identified in those samples with a retention time similar to that of sorbitol, but unfortunately mannitol and galactitol have an identical retention time to sorbitol using this method. By contrast, two other reports indicated that aldose reductase activity is not found in the human red cell hemolysate.[7,8] Instead, they described an oxidoreductase activity present in hemoglobin-free red cell preparations with the high glucose K_m characteristics of L-hexonate dehydrogenase (L-gulonate: NADP oxidoreductase, EC 1.1.1.19).[8]

This report presents evidence that intact human red cells do indeed accumulate intracellular sorbitol in response to the medium's glucose concentrations during in vitro incubations, and, after an 8 h fast, certain diabetics have significantly higher red cell sorbitol levels than nondiabetics, even when plasma glucose is in the normal range.

METHODS AND MATERIALS

Red cells from heparinized blood were routinely washed three times with isotonic saline at 4°C. Hemolysates were obtained by diluting washed packed cells with phosphate buffer, pH 7.4, followed by freezing and thawing three times.

For in vitro incubations, replicate 4.0 ml aliquots of washed packed cells were suspended in 10.0 ml of Kreb's bicarbonate, pH 7.4, and gassed with 5% CO_2 in air. All incubations were carried out at 37°C. Replicate flasks were

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identical, except that three different medium glucose concentrations were used (100, 300, and 500 mg/dl). To test the effect of medium glucose concentration on intracellular sorbitol accumulation, one hour incubations at three different glucose concentrations were performed. The effect of tetramethylene glutaric acid (TMG), a specific aldose reductase inhibitor,[9] on sorbitol accumulation was evaluated by incubating replicate red cell suspensions for 1,2, and 3 h in medium containing 500 mg/dl glucose with and without 0.1 mM TMG. At the end of incubation periods, red cells were sedimented by centrifugation, washed, and 1 vol of packed red cells was precipitated with 3 vol of cold 6% perchloric acid. The supernate was neutralized at 4°C with K_2CO_3 and used for sorbitol determination.

Heparinized blood samples from nondiabetic and insulin-dependent diabetic children were collected after an 8 to 10 h fast. The plasma was separated for glucose determination by the Beckman glucose analyzer. The red blood cells were washed, precipitated with perchloric acid, and neutralized as described above before the sorbitol determination.

Enzymatic measurement of sorbitol. Sorbitol was measured by a modification of the enzymatic assay of Clements.[14] Protein-free filtrate, 0.5 ml, was added to a reaction mixture, which consisted of 1.0 ml of 0.05 M glycine buffer, pH 9.4, containing 0.2 mM nicotinamide adenine dinucleotide (NAD) and 0.64 U of sorbitol dehydrogenase (Sigma Chemical Co., lot 94C-0237, from sheep liver). Blanks, with either filtrate, NAD, or sorbitol dehydrogenase deleted, were routinely run simultaneously. Sorbitol standards, ranging from 0.2 to 9.0 μg/ml (1.0 to 50.0 nmol/ml), analyzed by this technique produced a linear increase in fluorescence with a correlation coefficient of 0.99. Substrate exhaustion (9.0 μg/ml sorbitol) was complete in 25 min. The relative fluorescence due to NADH was measured in an Aminco SPF 125 spectrofluorimeter with an excitation wavelength of 366 nm and an emission wavelength of 452 nm. This procedure accurately measures as little as 0.06 μg/ml of sorbitol.

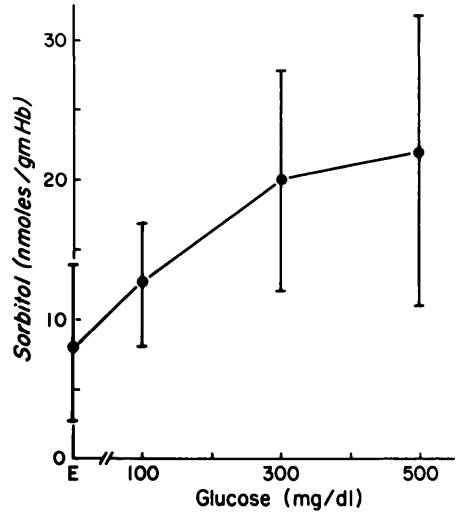
Gas-liquid chromatography measurement of sorbitol. Aldoses and alditols were analyzed as acetate derivatives. An internal standard, α-methylmannoside, was added to solutions of authentic standards and perchloric acid filtrates to obtain a final concentration of 5.0 μg/ml. After lyophilizing to dryness, the residue was mixed with 1.0 ml of pyridine and 0.2 ml of acetic anhydride and heated at 60°C for 1 h. The reaction was stopped by adding 0.5 ml of water, and the acetate derivatives were extracted into 0.5 ml of chloroform. Aliquots (1.0 μl) were analyzed isothermally at 200°C using a Perkin Elmer 900 gas chromatograph, equipped with a flame ionization detector and a 6 foot column containing 3% SP 2340 on 100/120 Supelcoport. Injector and manifold temperatures were 220° and 250°C, respectively. Identification and quantitation of sorbitol was achieved with the use of an automatic data-processing system (PEP-1, Perkin Elmer Corp). Peak retention times were recorded to the nearest $^1/_{100}$ of a minute, and relative retention times (RRT) were automatically determined based on the elution time of the internal standard, which was arbitrarily assigned an RRT of 1.000 (real time, 4.85 min). The ratio of sorbitol peak area to internal standard peak area showed a linear relationship to standard sorbitol concentration over the range from 5 to 50 μg contained in the original sample residue after lyophilization.

RESULTS AND DISCUSSION

Intact red cells from 10 nondiabetic individuals with a mean plasma glucose of 84.5 mg/dl accumulated intracellular sorbitol twofold to threefold above endogenous levels when incubated in medium containing glucose concentrations of 100, 300, and 500 mg/dl (5, 16, and 28 mM, respectively). These data, presented in Figure 1, also suggest that sorbitol accumulation tends to level off at glucose concentrations between 300 and 500 mg/dl. Although the identity and the physical characteristics of human red cell aldose reductase remain to be demonstrated, it seems reasonable that the oxidoreductase activity implied by these whole cell incubations is more consistent with the activity of mammalian aldose reductase (glucose K_m, 37 mM)[11] than with red cell L-hexonate dehydrogenase (glucose K_m, 396 mM).[7] Further evidence supporting the contention that human red cell sorbitol accumulation may be due to aldose reductase is presented in Figure 2. Intact red cells accumulate sorbitol linearly with time up to 3 h when incubated in medium containing glucose at 500 mg/dl. As shown in Figure 2, duplicate red cell suspensions containing 0.1 mM TMG show complete inhibition of this activity. TMG at 0.1 mM concentration has been reported to cause 68% inhibition of calf lens aldose reductase activity,[9] while the same concentration inhibits red cell L-hexonate dehydrogenase activity only slightly (9%).[8] The sorbitol accumulation in the intact human erythrocyte is completely blocked (100%) by 0.1 mM TMG. This indicates that L-hexonate dehydrogenase is not responsible for the sorbitol production noted in the intact red cell. TMG at 0.1 mM did not reduce intracellular glucose concentration (Table 1) and had no effect on the sorbitol dehydrogenase assay system used to quantitate sorbitol in these studies.

The sorbitol dehydrogenase used in these studies was tested and found to be highly specific for sorbitol. Xylitol, mannitol, galactitol, and inositol, at concentrations twofold to fourfold higher(100–200 nmol/ml) than the highest levels

FIGURE 1. The effect of medium glucose concentration on the production of sorbitol by intact human erythrocytes. Each point represents the mean sorbitol (nmol/g hemoglobin ± 2 SD) accumulation in red cells from 10 different nondiabetic individuals incubated for 1 h in the glucose concentration indicated. E represents the endogenous sorbitol content of the red cells before incubation. The mean plasma glucose concentration at the time the red cells were collected was 84.5 ± 14 mg/dl.



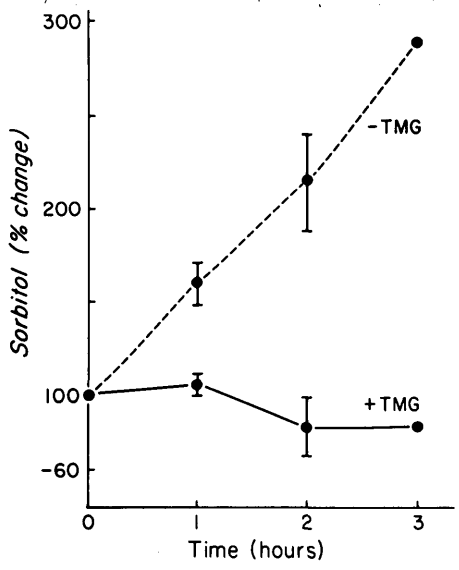


FIGURE 2. Increase of red cell sorbitol during in vitro incubation in 500 mg/dl glucose. Each point is the mean percent change in sorbitol content of red cells (nmol/g hemoglobin) from the preincubation level at time 0. Four separate incubations of red cells, collected from a single donor at one time, were carried out for 1, 2, and 3 h in media with 500 mg/dl glucose. Two incubations contained 0.1 mM tetramethylene glutaric acid (TMG), a specific aldose reductase inhibitor.

of sorbitol measured in these studies (50 nmol/ml), did not interfere with, or produce, a measureable increase in fluorescence.

The identity of the sorbitol measured enzymatically in these studies was confirmed by gas chromatographic analysis. Figure 3 (top) shows the elution patterns of the acetate derivatives obtained with a red cell filtrate as well as a standard mixture of polyols that also contained glucose. As seen in the red cell filtrate pattern, glucose is a predominate component which obscures sorbitol. Pretreatment of these

TABLE 1
Intracellular red cell sorbitol and glucose (influence of aldose reductase inhibitor)

Inhibitor	Sorbitol (nmol/ml) GLC	Sorbitol (nmol/ml) Enzyme	Glucose μmol/ml
No	48.4	47.7	2.9
No	83.4	75.3	2.9
Yes	28.8	27.0	5.3
Yes	30.2	29.1	3.3

Intracellular sorbitol and glucose concentrations found in replicate erythrocyte samples incubated for 3 h in Kreb's bicarbonate, pH 7.4, containing glucose (500 mg/dl). The aldose reductase inhibitor, tetramethylene glutaric acid (0.1 mM), was added to the incubation system where indicated.

samples with glucose oxidase permits accurate identification and quantitation of sorbitol (Figure 3, bottom). While treatment with glucose oxidase produces a few, currently unidentified peaks elsewhere in the chromatogram, none of these interferes with the analysis of sorbitol. Red cell filtrates with sorbitol, determined enzymatically to be greater than 5.0 μg/ml, were found only in diabetics. The presence of sorbitol in several of these filtrates was confirmed by gas chromatography analysis after glucose oxidase treatment, as demonstrated in Figure 3. Sorbitol, measured in the same filtrates both enzymatically and by gas chromatography, agree with less than 10% error (Table 1).

The formation of sorbitol in response to a low glucose concentration strongly suggests the presence of an oxidoreductase activity in human red cells similar to that of aldose reductase. Hemolysates of the same red cells found to accumulate sorbitol were mixed with Kreb's bicarbonate, pH 7.4, containing 5 mM mercaptoethanol, 0.2 mM NADPH, and 28 mM glucose, and were incubated up to 1 h at 37°C. No sorbitol formation could be detected in these cell-free mixtures. We currently cannot explain why the oxidoreductase

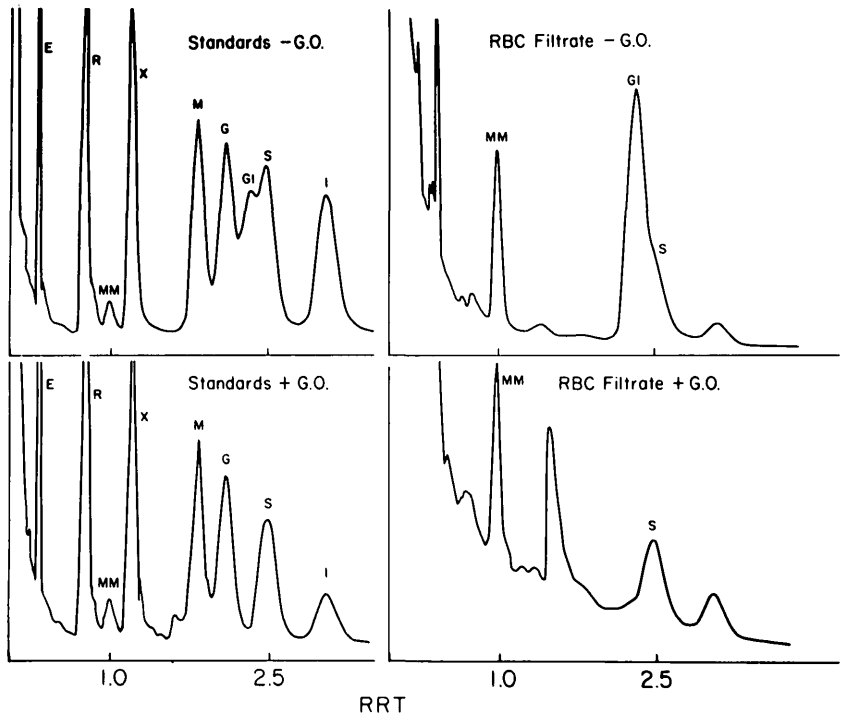


FIGURE 3. Gas-liquid chromatograms of acetate derivatives of *E*-erythritol, *R*-ribitol, *MM*-αmethylmannoside, X-xylitol, *M*-mannitol, *G*-galactitol, *GL*-glucose, *S*-sorbitol, and *I*-inositol separated on a 6 foot column of 3% SP 2340 on 100/120 Supelcoport isothermally at 200°C with a carrier gas flow rate of 40.0 ml/min. MM was assigned an RRT of 1.000 (real time, 4.85 min). The chromatograms in the upper panel were run on unmodified original samples; the samples in the lower panel were treated with glucose oxidase (GO) before acetylation to remove interfering glucose. The concentrations of each of the polyols and glucose in the standard was 50.0 μg/ml; the concentration of MM in each chromatogram was 5.0 μg/ml. The concentration of sorbitol in the red cell filtrate depicted in this figure was 83.5 nmol/ml. The sorbitol concentration measured in the same sample with sorbitol dehydrogenase was 77.0 nmol/ml. The chromatogram of the red cell sample demonstrates the interference of glucose on sorbitol quantitation by this method.

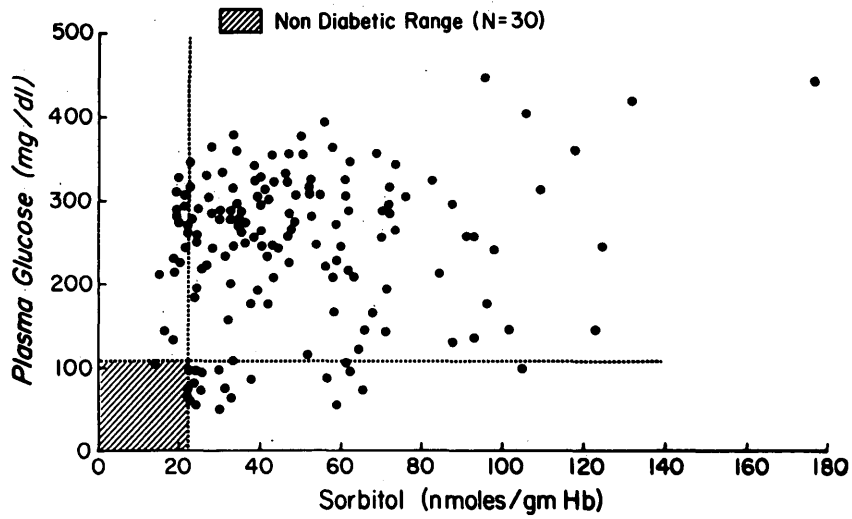


FIGURE 4. Red cell sorbitol (nmol/g hemoglobin) and plasma glucose (mg/dl) in blood samples collected from children with diabetes after fasting for 8 h. The shaded area represents the range for fasting nondiabetic children.

activity clearly present in intact cells appears to be lost in the cell-free lysates. Cellular disruption could alter the milieu and inhibit aldose reductase or increase the activity of sorbitol dehydrogenase enough to obscure sorbitol production. Loss and/or alterations of activity have been described for another red cell enzyme (glucose-6-phosphate dehydrogenase) following hemolysis.[12] Manipulation of substrate, cofactors, and buffers in that case was required to demonstrate enzyme activity in hemolysates that was quite evident in the intact cell. A similar situation may exist for red cell aldose reductase and may explain why other investigators[7,8] have not been able to demonstrate the presence of aldose reductase activity in broken red cells.

These data support the hypothesis that intact human red cells are capable of converting glucose to sorbitol, and the amount of intracellular sorbitol may be a reflection of the surrounding glucose concentration and/or flux through the polyol pathway. It, therefore, seems reasonable that the red cell sorbitol levels found in diabetes may be of interest.

Red cell sorbitol, measured in 155 children with diabetes (28.0 ± 18.9 nmol/g hemoglobin) after at least an 8 h fast, was significantly higher ($P < 0.001$) than the levels found in red cells from 30 nondiabetics (10.1 ± 7.2 nmol/g hemoglobin) ($\bar{x}$ ± SD). Figure 4 shows a plot of these data against the coincident plasma glucose concentration. Linear regression analysis shows statistical significance ($r = 0.59$, slope = 2.61, intercept = 90.0, $P < 0.001$); however, it is evident that both high red cell sorbitol with normal plasma glucose as well as normal red cell sorbitol with high plasma glucose are represented in portions of this diabetic population even after fasting. Thus, some factor in addition to glucose concentration influences intracellular sorbitol levels. This observation is presently being evaluated by sequential samples in the same individuals.

If the accumulation of intracellular sorbitol is indeed one of the factors contributing to the pathologic consequences of diabetes mellitus, then monitoring the tissue level of this substance should be important for preventing these complications. The tissues that manifest most of the long-term complications of diabetes are the lens, nerve, and blood vessels. These tissues are freely permeable to glucose, and insulin is not required for intracellular transport. The intracellular glucose and sorbitol levels vary directly with the extracellular glucose concentration. These are features characteristic of the human erythrocyte. Moreover, the red cell is more readily available for sampling. Red cell sorbitol, therefore, may be a useful indicator of the tissue sorbitol levels that participate in the pathogenesis of diabetes-associated complications.

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