Crucial Role of Aldose Reductase Activity and Plasma Glucose Level in Sorbitol Accumulation in Erythrocytes From Diabetic Patients

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Increased sorbitol levels have been demonstrated in tissues of diabetic patients. Although tissue sorbitol levels correlate with plasma glucose levels, a large variability in sorbitol levels has been observed among diabetic patients with similar plasma glucose levels. This variability in tissue sorbitol levels may be due to differences in the activity of aldose reductase, the enzyme that converts glucose to sorbitol. In this study, we isolated aldose reductase from erythrocytes of 31 diabetic patients and 6 nondiabetic control subjects, measured its activity, and compared it to simultaneously measured erythrocyte sorbitol levels. The activity of erythrocyte aldose reductase was increased in diabetic patients compared with control subjects (28.1 ± 1.4 vs. 22.4 ± 1.7 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $P < 0.05$), but there was an approximately threefold variation in aldose reductase activity among diabetic patients. Erythrocyte aldose reductase activity and fasting plasma glucose levels significantly correlated with the erythrocyte sorbitol level in all individuals ($r = 0.48$, $P < 0.005$ and $r = 0.63$, $P < 0.005$, respectively). The sorbitol level was higher in patients with high aldose reductase activity than in those who had low enzyme activity for any given level of glycemia. The sorbitol production rate calculated from K_m and V_{max} values showed a better correlation with the erythrocyte sorbitol level ($r = 0.80$, $P < 0.005$), and there was also a good correlation between the erythrocyte sorbitol level and the product of aldose reductase activity by plasma glucose level ($r = 0.70$, $P < 0.005$). We conclude that both aldose reductase activity and the ambient plasma glucose concentration are important determinants of the level of sorbitol that accumulates in tissues of diabetic patients. *Diabetes* 40:1233–40, 1991

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The accumulation of polyols in tissues has been implicated in the etiology of diabetic complications (1–4). Although increased sorbitol levels have been demonstrated in tissues of diabetic patients (5–7), the mechanism by which the tissue sorbitol level is increased has not been completely elucidated. It is known that an elevation of the plasma glucose level results in an increase in the erythrocyte sorbitol level (8–10), and there is a correlation between the ambient plasma glucose level and erythrocyte sorbitol level in diabetic patients (11–13). However, there is marked variability in the erythrocyte sorbitol level seen among patients who have a similar level of glycemia (12). This variability suggests that there may be factors other than the ambient plasma glucose level that determine tissue sorbitol levels.

It seems reasonable to think that the activity of aldose reductase, the first enzyme of the polyol pathway that converts glucose to sorbitol, may affect the tissue sorbitol level. It is well known that enzyme activity and substrate concentration determine the production rate in any enzymatic reaction.

Several studies have shown that aldose reductase expression is increased in some tissues of diabetic subjects (14,15). In addition, the inhibitors of aldose reductase have been reported to decrease tissue sorbitol levels (12,16–18). These observations indicate that high levels of aldose reductase and the ambient plasma glucose level may be responsible for the accumulation of sorbitol in tissues of diabetic subjects.

The relationship between aldose reductase activity and the sorbitol level in diabetic patients, however, has not been clarified. To better understand tissue sorbitol accumulation, it seems important to determine whether the variability of sorbitol levels observed in previous studies (11–13) is caused by differences in aldose reductase activity among diabetic patients. If aldose reductase activity varies widely among diabetic patients and correlates with the tissue sor-

bitol level, patients with high aldose reductase activity may accumulate more sorbitol in their tissues for any given level of glycemia. Thus, these individuals may be more susceptible to diabetic complications than those who have low enzyme activity.

To clarify whether aldose reductase activity is increased in diabetic patients and whether it varies among individuals and correlates with tissue sorbitol levels, we isolated aldose reductase from erythrocytes of diabetic patients and nondiabetic control subjects and compared its activity with simultaneously determined erythrocyte sorbitol levels.

RESEARCH DESIGN AND METHODS

Blood samples were obtained from 31 diabetic patients and 6 nondiabetic individuals after a 10- to 12-h overnight fast. Consent to collect specimens was obtained from all individuals. The clinical data of the subjects are shown in Table 1. HbA_{1c} was measured with high-performance liquid chromatography as described previously (19).

Aldose reductase was isolated from erythrocytes of diabetic patients by a modification of the procedure described by Das and Srivastava (20). The entire procedure was carried out at 0–4°C. Approximately 35 ml of blood was drawn from each diabetic patient into a heparin vacutainer and centrifuged for 15 min at 1000 × *g*. After removing the buffy coat by suction, the erythrocytes were suspended in 3 vol of phosphate-buffered saline (100 mM potassium phosphate/0.145 M NaCl [9:1 vol/vol]) and centrifuged again. This step was then repeated twice. Fifteen milliliters of the washed erythrocytes was stored at −20°C and analyzed within 3 wk.

After thawing, the erythrocytes were hemolyzed with an equal volume of 10 mM imidazole-HCl buffer containing 5 mM 2-mercaptoethanol, pH 7 (buffer 1), by a Heat System-Ultrasonics model 185F Sonicator cell disruptor (Plain View, NY). The hemolysate was dialyzed twice against 60 vol buffer 1 for 2.5 h and then centrifuged at 40,000 × *g* for 30 min. The resulting supernatant was applied to a DEAE-cellulose (DE52) column (1 × 8 cm) that had been preequilibrated with buffer 1. After washing the column with 3 vol buffer 1, the enzyme was eluted with a linear gradient of 0–400 mM NaCl. Twenty-milliliter fractions were collected and then dialyzed overnight against 100 vol of 25 mM imidazole-HCl buffer containing 5 mM 2-mercaptoethanol, pH 7.4 (buffer 2).

The dialysate was then loaded on a PBE 94 column (1 × 13 cm) preequilibrated with buffer 2. The column was washed with 3 vol of buffer 2 and eluted with Polybuffer 74 (diluted 1:8, pH 4) with a fraction volume of 2 ml at flow rate of 20 ml/h. The enzyme activity of aldose reductase was measured in each fraction and summed to obtain total enzyme activity. The enzyme activity was expressed as units per gram of hemoglobin by dividing the total activity by total hemoglobin amount contained in an original sample.

Aldose reductase was assayed by the method described by Das and Srivastava (20). The enzyme activity was measured spectrophotometrically at 340 nm. The enzyme activity was expressed as the amount of NADPH that was oxidized per minute at 37°C. The assay mixture contained 50 mM potassium phosphate, 5 mM 2-mercaptoethanol, 0.4 M lithium sulfate, 10 mM DL-glyceraldehyde, 0.1 mM NADPH, and 0.1 ml enzyme solution. The absorbance at 340 nm was recorded on a Carry model 118 recording spectrophotometer (Palo Alto, CA) for 5 min.

Protein concentration of partially purified extracts was determined by the procedure described by Bradford (21), with bovine serum albumin as a standard.

Hemoglobin concentration was determined by the methods previously described by Drabkin and Austin (22).

Erythrocyte sorbitol levels were determined by the methods described by Liao et al. (23). In brief, blood was drawn into EDTA vacutainers and centrifuged at 1000 × *g* for 15 min at 4°C. After removing the plasma and buffy coat, the blood was suspended in 2 vol saline and centrifuged again. The packed erythrocytes were diluted with 2 vol saline and stored at −70°C until use.

After thawing, 1.5 ml of the diluted erythrocyte aliquots was taken and mixed with 2 ml of cold chloroform. The sample was centrifuged at 1000 × *g* for 1 min, and 2 ml of 5.8% perchloric acid was added to precipitate the proteins. After centrifugation at 1000 × *g* for 5 min, 1 ml of the resulting supernatant was pipetted and mixed with 1 ml of 0.5 M tribasic sodium phosphate–HCl buffer, pH 11. The sample was then mixed with 1 ml of NAD solution containing 2.4 mM NAD and 47 mM magnesium acetate and with 1 ml of 6 U/ml L-iditol dehydrogenase solution. The mixture was incubated at 37°C for 25 min. The formation of NADH was measured by the change in fluorescence at an emission/excitation wavelength of 455/343 nm by a model LS 3 spectrofluorophotometer (Perkin-Elmer, Norwalk, CT).

DE52 was purchased from Whatman Bio System (Maidstone, UK). PBE 94 and Polybuffer 74 were purchased from Pharmacia (Uppsala, Sweden). Protein assay was obtained from Bio-Rad (Richmond, CA). Perchloric acid, tribasic sodium phosphate, and magnesium acetate were purchased from Baker Chemical (Phillipsburg, NJ). The other chemicals were purchased from Sigma (St. Louis, MO).

TABLE 1
Clinical data of diabetic and nondiabetic individuals

	n	Age (yr)	IDDM	NIDDM	Duration of diabetes (yr)	Fasting plasma glucose (mM)	HbA_{1c} (%)
Diabetic	23 M/8 F	50 ± 2	12	19	12 ± 2	10.4 ± 0.9	6.3 ± 0.2
Nondiabetic	6 F	30 ± 2				4.7 ± 0.2	4.1 ± 0.1

Values are means ± SE. IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.

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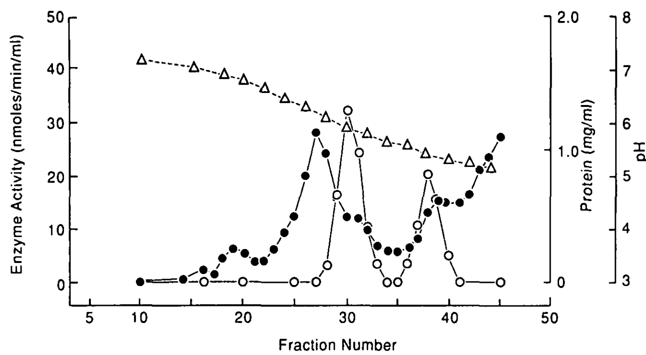

FIG. 1. Chromatofocusing of human erythrocyte aldose reductase and aldehyde reductase by PBE 94 column chromatography. ○, Enzyme activity; ●, protein profile; △, pH.

RESULTS

Table 1 gives the clinical data of diabetic and nondiabetic individuals we studied. Of the 31 patients, 23 were men, and 8 were women. Twelve had insulin-dependent and 19 had non-insulin-dependent diabetes mellitus. The mean ± SE fasting plasma glucose level was 10.4 ± 0.9 mM, and the HbA_{1c} level averaged 6.3 ± 0.2% (normal range 3.7–5.1%).

Figure 1 shows the separation of aldose reductase from aldehyde reductase after PBE 94 column chromatography. The first enzyme peak, eluted at pH ~5.9, was identified as aldose reductase by its biochemical characteristics. This enzyme had a relatively low affinity for D-glucose and a high affinity for glyceraldehyde (Table 2). This enzyme also utilized D-xylose and D-glucuronate as substrates. The K_m and relative V_{max} values for these substrates were similar to those reported previously for aldose reductase (20,24–28). Table 3 shows the effect of sulfate on the enzyme activity. Both lithium sulfate and ammonium sulfate stimulated the activity of the first enzyme peak. This has also been reported to be characteristic of aldose reductase (20,24,26,28).

The second enzyme peak (Fig. 1) eluted at pH ~5.4. This enzyme had a substrate specificity similar to those previously reported for aldehyde reductase (27–29), aldehyde reductase II (20), or hexonate dehydrogenase (25) (Table 2). Sulfate has been reported to suppress (20) or stimulate (28) the activity of aldehyde reductase. We observed a mild stimulation of the activity of this enzyme by the addition of sulfate (Table 3).

Figure 2 shows erythrocyte sorbitol levels in diabetic and nondiabetic individuals. Erythrocyte sorbitol level was significantly higher in diabetic patients than in nondiabetic control subjects (20.0 ± 2.3 vs. 15.5 ± 3.1 nmol/g Hb, $P < 0.025$). However, there was a more than sixfold difference in the sorbitol levels among diabetic patients (9.0–63.9 nmol/g Hb).

As shown in Fig. 3, the activity of erythrocyte aldose reductase was significantly increased in the diabetic patients compared with nondiabetic control subjects (28.1 ± 1.4 vs. 22.4 ± 1.7 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $P < 0.05$). The enzyme activity, however, varied more than threefold among diabetic individuals (14.6–44.3 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb).

Figure 4 shows the relationship between the plasma glucose and erythrocyte sorbitol level in diabetic and nondiabetic individuals. There was a significant correlation between the plasma glucose and erythrocyte sorbitol level ($r = 0.63$, $P < 0.005$). It is obvious, however, that there was a great deal of variability in the erythrocyte sorbitol levels

TABLE 2
Substrate specificity of human erythrocyte aldose reductase and aldehyde reductase

Substrate	Aldose reductase		Aldehyde reductase	
	K_m (mM)	Relative V_{max} (%)	K_m (mM)	Relative V_{max} (%)
DL-Glyceraldehyde	0.125	100	6.9	100
D-Glucose	137	54	1075	19
D-Xylose	19.6	84	769	52
D-Glucuronate	11.1	54	7.4	135

TABLE 3
Effects of sulfate on activity of aldose reductase and aldehyde reductase

Sulfate	Aldose reductase (%)	Aldehyde reductase (%)
Baseline	100	100
0.4 M $LiSO_4$	284	138
0.4 M $(NH_4)_2SO_4$	234	140

DL-Glyceraldehyde (10 mM) was used as substrate.

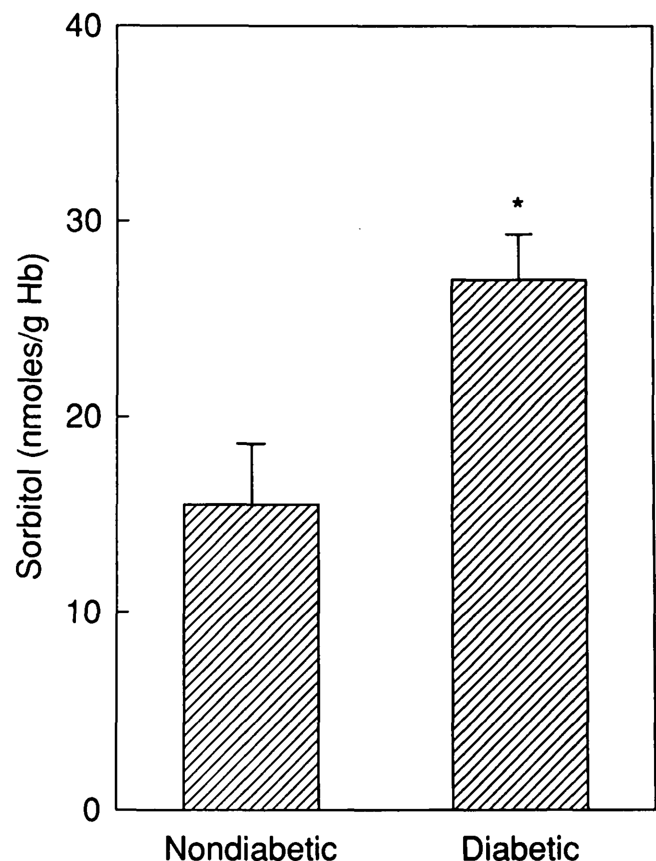

FIG. 2. Mean ± SE erythrocyte sorbitol levels in diabetic and nondiabetic individuals. *$P < 0.025$.

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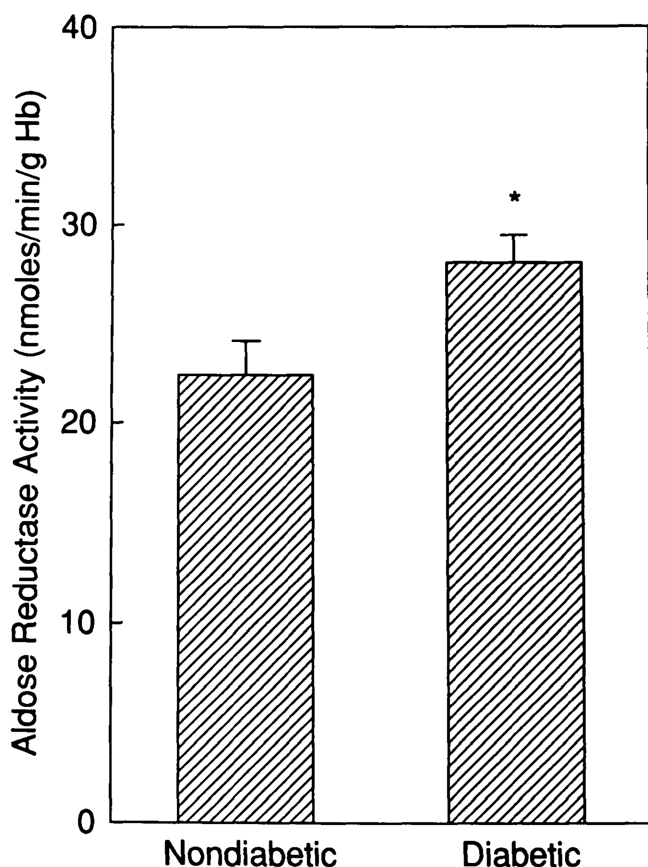

FIG. 3. Mean ± SE erythrocyte aldose reductase activity in diabetic and nondiabetic individuals. *$P < 0.05$.

among individuals whose levels of glycemia were quite similar. In fact, there was no significant correlation between the plasma glucose and the erythrocyte sorbitol level in the subsets of patients whose plasma glucose levels were <8 mM or >8 mM ($r = 0.33$ and $r = 0.42$, respectively).

Figure 5 shows the relationship between the erythrocyte sorbitol level and the erythrocyte aldose reductase activity in all individuals, including nondiabetic control subjects. Aldose reductase activity varied approximately threefold among the diabetic patients, and there was a significant correlation between the enzyme activity and the sorbitol level ($r = 0.48$, $P < 0.005$). We looked at this same relationship in subsets of these individuals who had high (>8 mM) or low (<8 mM) levels of glycemia, in which subsets, the erythrocyte sorbitol level did not significantly correlate with plasma glucose level. As can be seen in Fig. 6, there was a significant correlation between the two in both groups of patients ($r = 0.47$, $P < 0.05$ and $r = 0.55$, $P < 0.025$, respectively).

Figure 7 shows the relationship between erythrocyte sorbitol and plasma glucose levels in two subsets of the individuals in whom erythrocyte aldose reductase activity was high (>27 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $n = 18$) or low (<27 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $n = 19$). In both groups, sorbitol levels significantly correlated with plasma glucose levels ($r = 0.61$, $P < 0.01$ and $r = 0.49$, $P < 0.05$, respectively). As is clear in the figure, erythrocyte sorbitol levels were higher in individuals who had high aldose reductase activity than those who had low activity of the enzyme given similar levels of glycemia. In addition, it is apparent that the accumulation of erythrocyte sorbitol as plasma glucose increases was steeper in the group with high enzyme activity than in those with low activity (Δ sorbitol and Δ plasma glucose = 1.04 and 0.85, respectively).

We determined K_m and V_{max} values for glucose and glyceraldehyde in 11 diabetic and 3 control subjects (Table 4). We calculated sorbitol production rate based on the Michaelis-Menten equation with the kinetic data obtained in each individual as follows

$$v = \frac{V_m(G) \times [\text{glucose}]}{(K_m + [\text{glucose}])} = \frac{\text{relative } V_{max} \times V_m(GA) \times [\text{glucose}]}{(K_m + [\text{glucose}])}$$

where $V_m(G)$ and $V_m(GA)$ indicate V_{max} for glucose and glyceraldehyde, respectively, and K_m and [glucose] indicate K_m for glucose and concentration of glucose, respectively.

The activity measured with 10 mM of glyceraldehyde, which is a concentration >50 times higher than the K_m for the enzyme, is very close to the V_{max} for glyceraldehyde. The glucose concentration in erythrocytes is thought to approximate the plasma glucose level (8,30,31). Thus, the sorbitol production velocity would be given by the following equation

$$v = \frac{\text{relative } V_{max} \times \text{measured activity} \times [\text{plasma glucose}]}{K_m + [\text{plasma glucose}]}$$

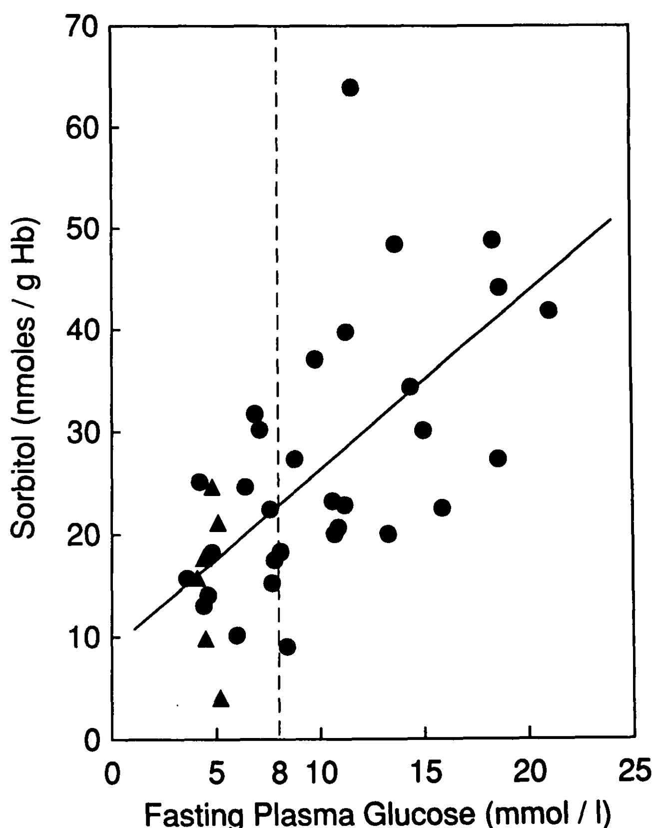

FIG. 4. Relationship between fasting plasma glucose level and erythrocyte sorbitol level in diabetic (●) and nondiabetic (▲) individuals. $r = 0.63$, $P < 0.005$.

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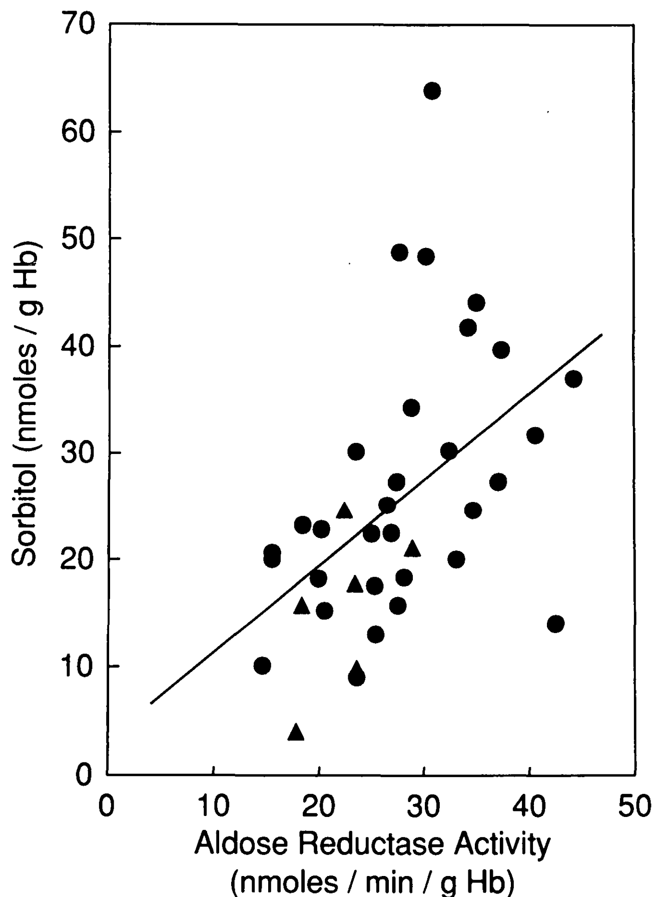

FIG. 5. Relationship between erythrocyte sorbitol level and aldose reductase activity in diabetic (●) and nondiabetic (▲) individuals. $r = 0.48$, $P < 0.005$.

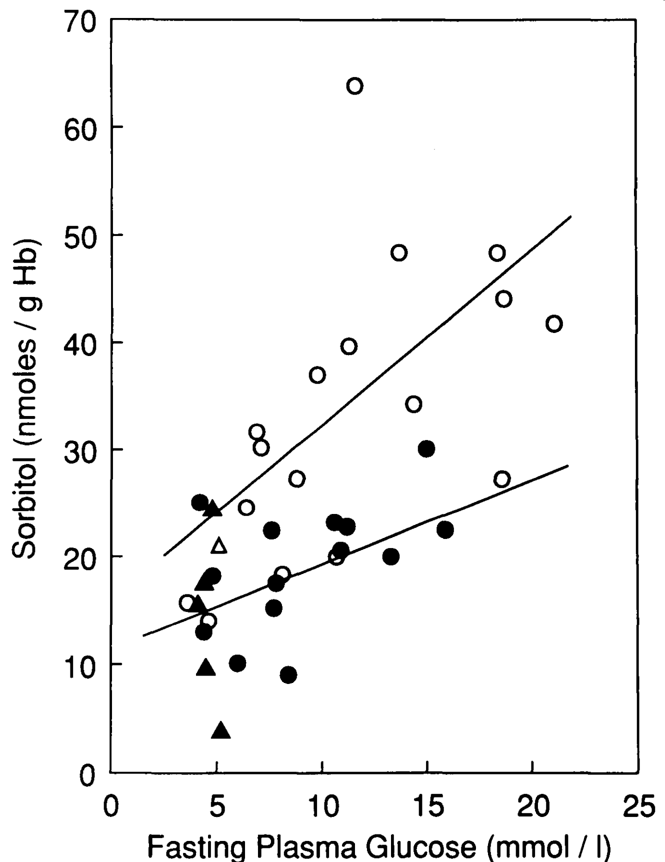

FIG. 7. Effect of aldose reductase activity on relationship between erythrocyte sorbitol and plasma glucose level. ○ and ●, data from diabetic patients with high (>27 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $r = 0.61$, $P < 0.01$) and low (<27 $nmol \cdot min^{-1} \cdot g^{-1}$ Hb, $r = 0.49$, $P < 0.05$) enzyme activity, respectively. △ and ▲, data from nondiabetic individuals with high and low enzyme activity, respectively.

Figure 8 shows the relationship between the calculated sorbitol production velocity and erythrocyte sorbitol level. The sorbitol production rate was highly correlated with the erythrocyte sorbitol level ($r = 0.80$, $P < 0.005$).

The product of aldose reductase activity and the plasma glucose level was calculated in all individuals. As shown in Fig. 9, there was a better correlation between erythrocyte sorbitol level and the product ($r = 0.70$, $P < 0.005$) than that between the erythrocyte sorbitol level and the plasma glucose level ($r = 0.63$) or enzyme activity ($r = 0.48$).

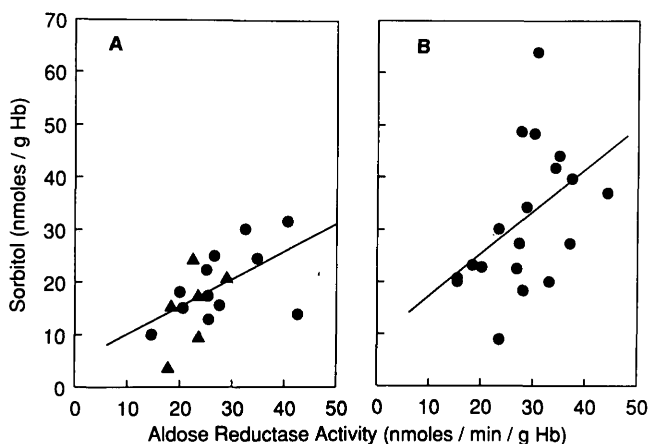

FIG. 6. Erythrocyte sorbitol levels and aldose reductase activity in diabetic (●) and nondiabetic (▲) individuals whose glycemic levels are <8 mM ($r = 0.55$, $P < 0.025$; *A*) or >8 mM ($r = 0.47$, $P < 0.05$; *B*).

DISCUSSION

We isolated aldose reductase from the erythrocytes of diabetic and nondiabetic subjects and compared its activity with the erythrocyte sorbitol concentration. Our results showed that the activity of erythrocyte aldose reductase was increased in diabetic patients compared with nondiabetic subjects, but there was a large difference in the enzyme activity among patients. The enzyme activity and plasma glucose level were correlated with the erythrocyte sorbitol level measured simultaneously, and the sorbitol content was higher in patients with high aldose reductase activity than in those who had low enzyme activity at any given level of glycemia. The sorbitol production rate, calculated from measured K_m and V_{max} values, also showed a good correlation with the sorbitol level. Although observed K_m and V_{max} values varied approximately twofold among individuals, it is difficult to conclude from these results that these differences actually should be interpreted as meaning there is variability in enzyme kinetics among individuals. Given the present purity of our enzyme mixture, we are not prepared to make such a claim. Complete purification would be necessary to appropriately address this issue.

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TABLE 4
K_m and V_{max} values in diabetic and nondiabetic subjects

	n	K_m (mM) Glucose	K_m (mM) Glyceraldehyde	Relative V_{max}
Diabetic	11	102 ± 12	0.102 ± 0.008	0.53 ± 0.05
Nondiabetic	3	115 ± 9	0.116 ± 0.009	0.32 ± 0.03

Values are means ± SE. Relative V_{max} was calculated as V_{max} for glyceraldehyde = 1. For nondiabetic subjects, an approximately double-concentrated enzyme solution was used because of low enzyme activity.

We also showed that the product of aldose reductase activity and the plasma glucose level correlated better with the erythrocyte sorbitol level than either the enzyme activity or plasma glucose level. These results seem to agree with the well-known theory that production velocity in any enzymatic reaction is proportional to an enzyme amount and a substrate concentration in cases where the enzyme has the same kinetics and the substrate concentration is much less than K_m. Although it remains to be seen, as mentioned above, whether the kinetics of aldose reductase is the same among individuals, our observation indicates that the product of aldose reductase activity and the plasma glucose level may be a better indicator of tissue sorbitol accumulation than the plasma glucose level alone.

However, there are differences in erythrocyte sorbitol levels among individuals in whom the products or the calculated sorbitol production rate are similar. This fact indicates that some factors other than aldose reductase activity and the plasma glucose level may affect sorbitol level. One possibility to explain these differences is the activity of L-iditol dehydrogenase, the second enzyme of the polyol pathway. It has been reported that the activity of this enzyme is lowered whereas aldose reductase activity is increased in diabetic rat lens (32). If the activity of L-iditol dehydrogenase varies to a large extent among diabetic patients, it seems possible that the difference in this enzyme's activity could affect tissue sorbitol levels.

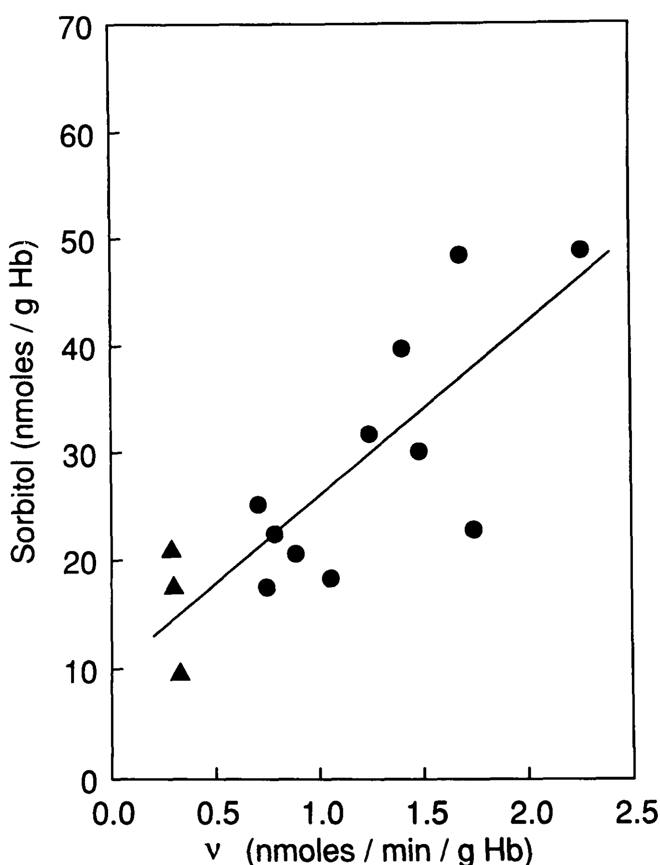

FIG. 8. Relationship between erythrocyte sorbitol level and calculated sorbitol production rate (ν) in diabetic (●) and nondiabetic (▲) individuals. r = 0.80, P < 0.005.

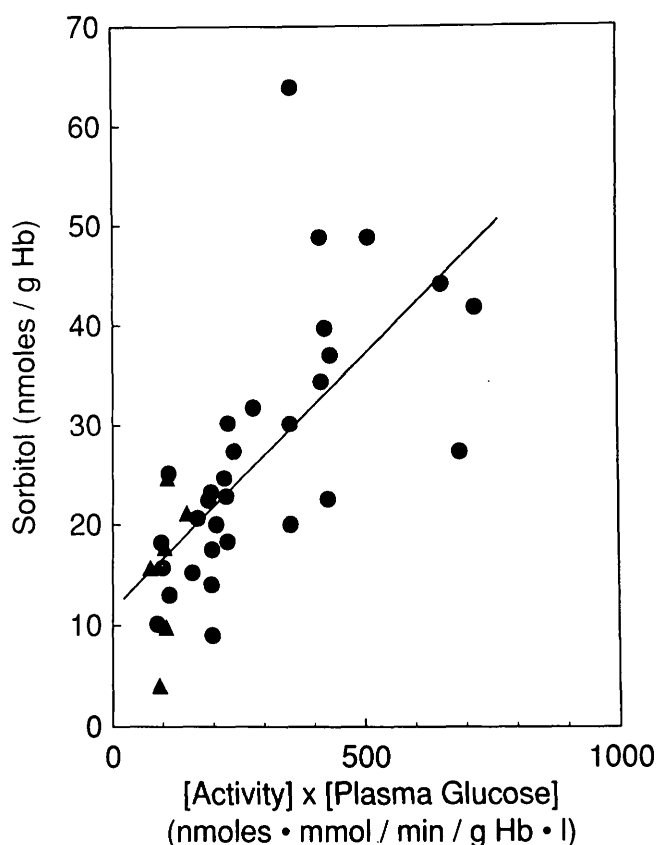

FIG. 9. Relationship between erythrocyte sorbitol level and product of aldose reductase activity by plasma glucose concentration in diabetic (●) and nondiabetic (▲) individuals. r = 0.70, P < 0.005.

The accumulation of sorbitol as a consequence of aldose reductase activity in tissues of diabetic subjects has been suggested as an important factor in the pathogenesis of diabetic complications (3,4,33). The observations that aldose reductase inhibitors are able to reduce tissue sorbitol levels (12,16–18,34) and to prevent or ameliorate diabetic complications in experimental diabetes in animals and human diabetes (35–42) support this concept.

The activity of aldose reductase, however, had not been directly measured in diabetic patients. It was not clear whether the enzyme activity was increased in diabetic patients and whether there were differences in the enzyme activity among diabetic patients. In addition, no direct evidence demonstrating the correlation between aldose reductase activity of a specific tissue and that tissue's sorbitol level had ever been obtained.

Our results indicate that erythrocyte aldose reductase activity is increased in diabetic patients yet varies to a large

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extent among individuals and that the aldose reductase activity for any given diabetic individual is as important as the plasma glucose level in determining tissue sorbitol levels. Thus, the activity of aldose reductase could be a crucial factor in the pathogenesis of diabetic complications. Patients who have high aldose reductase activity would be expected to produce more sorbitol in their tissues than those with low enzyme activity given an equal level of glycemia and thus may be more susceptible to diabetic complications than those who have a low level of enzyme activity given equal glycemic control. The recent report of the correlation between diabetic complications and the erythrocyte sorbitol–blood glucose ratio (13), which is thought to relate to erythrocyte aldose reductase activity, seems to support this hypothesis.

Approximately 20–25% of diabetic patients regardless of diabetic control never get severe complications of their diabetes, even if they have diabetes for many years. Another 5% of patients get severe diabetic complications after having the disease for only a short time and sometimes with rather well-controlled diabetes (43,44).

The variability in aldose reductase activity among diabetic patients and its relationship with sorbitol accumulation we have reported in this article might be involved in these curious differences in the susceptibility among patients to diabetic complications. The direct relationship between aldose reductase activity and diabetic complications, however, remains to be demonstrated.

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