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Activated and unactivated forms of human erythrocyte aldose reductase

(aldose reductase activation/diabetes complications)

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ABSTRACT **Aldose reductase (alditol:NADP$^+$ 1-oxidoreductase, EC 1.1.1.21) has been partially purified from human erythrocytes by DEAE-cellulose (DE-52) column chromatography. This enzyme is activated severalfold upon incubation with 10 μM each glucose 6-phosphate, NADPH, and glucose. The activation of the enzyme was confirmed by following the oxidation of NADPH as well as the formation of sorbitol with glucose as substrate. The activated form of aldose reductase exhibited monophasic kinetics with both glyceraldehyde and glucose (K_m of glucose = 0.68 mM and K_m of glyceraldehyde = 0.096 mM), whereas the native (unactivated) enzyme exhibited biphasic kinetics (K_m of glucose = 9.0 and 0.9 mM and K_m of glyceraldehyde = 1.1 and 0.14 mM). The unactivated enzyme was strongly inhibited by aldose reductase inhibitors such as sorbinil, alrestatin, and quercetrin, and by phosphorylated intermediates such as ADP, glycerate 3-phosphate, glycerate 1,3-bisphosphate, and glycerate 2,3-trisphosphate. The activated form of the enzyme was less susceptible to inhibition by aldose reductase inhibitors and phosphorylated intermediates.**

Aldose reductase (alditol:NADP$^+$ 1-oxidoreductase, EC 1.1.1.21) has been implicated in the etiology of complications of diabetes such as neuropathy, nephropathy, retinopathy, and cataractogenesis (1–4). This enzyme reduces glucose to sorbitol in the presence of NADPH. The major cause of the complications of diabetes could be the change in the osmotic pressure due to the accumulation of sorbitol, which is relatively impermeable through biological membranes (5). Increased levels of sorbitol in the tissues of diabetic subjects and in diabetic animals have indeed been reported (6–9). In addition, incubation of human erythrocytes with increasing concentrations of glucose (30–50 mM) results in increased levels of sorbitol in the erythrocytes (10, 11). The role of aldose reductase in the formation of sorbitol in the erythrocyte was further confirmed by lower levels of erythrocyte sorbitol in diabetic subjects receiving aldose reductase inhibitors such as sorbinil and alrestatin as compared with diabetic subjects receiving a placebo (12, 13). Contrary to earlier reports that human erythrocytes contain only L-hexonate dehydrogenase (aldehyde reductase II, EC 1.1.1.2), which does not use glucose as substrate, we have shown the presence of aldose reductase in human erythrocytes and have purified it to homogeneity (14, 15). The kinetic, immunological, and structural properties of purified erythrocyte aldose reductase were similar to lens aldose reductase. The erythrocyte aldose reductase does not crossreact with antiserum against aldehyde reductase II (14, 15). We now demonstrate that under physiological conditions, aldose reductase exhibits biphasic kinetics (K_m glucose = 9 and 0.9 mM) because it exists in the activated and unactivated forms. The activated form of the enzyme reduces glucose to sorbitol with a higher efficiency than does the unactivated form. The unactivated enzyme can be activated by incubation with 10 μM each of glucose 6-phosphate (Glc-6-*P*), NADPH, and glucose. Furthermore, the activated enzyme is less susceptible to inhibition by aldose reductase inhibitors as compared with the unactivated form of the enzyme.

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MATERIALS AND METHODS

Blood from healthy volunteers, fasting overnight, was drawn in heparinized vacutainers and used immediately. DEAE-cellulose (DE-52) was purchased from Whatman Chemical, Kent, UK, and sorbitol dehydrogenase (sheep liver), Glc-6-*P* dehydrogenase (Baker's yeast, type VII), and all other chemicals used in the present study were purchased from Sigma. Sorbinil and alrestation were supplied by Pfizer and Ayrest, respectively.

Partial Purification of Aldose Reductase. Erythrocytes from whole blood were isolated, washed, and hemolyzed as described earlier (14). Unless otherwise mentioned, all of the operations were carried out at 4°C. Erythrocytes were hemolyzed with 4 vol of buffer A (10 mM potassium phosphate, pH 7.0/5 mM 2-mercaptoethanol), dialyzed twice against 50 vol of the same buffer, and centrifuged at 10,000 × *g* for 30 min. The supernatant was passed through a DE-52 column (2.5 × 80 cm), preequilibrated with buffer A, at 15 ml per hour. The column was washed with 5–8 column volumes of buffer A, and the elution of the enzyme was performed by a 2-liter gradient of 0–200 mM NaCl in buffer A. The fractions containing aldose reductase activity were pooled, dialyzed against buffer A, and analyzed for cross-contamination with aldehyde reductase II by using immunological and kinetic methods as described earlier (14). The pooled fraction containing aldose reductase essentially free from aldehyde reductase II was used for all subsequent studies.

Enzyme Activity Determination. Aldose reductase activity was determined as described (14). Briefly, the reaction mixture in a 1-ml system contained 50 mM potassium phosphate (pH 6.0), 5 mM 2-mercaptoethanol, 0.1 mM NADPH, 0.4 M Li_2SO_4, 10 mM DL-glyceraldehyde or 5 mM α-D-glucose, and an appropriate amount of the enzyme. The reaction was started by the addition of DL-glyceraldehyde or glucose, and the decrease in optical density at 340 nm was recorded with a Gilford spectrophotometer model 2600. One unit of enzyme activity is defined as the amount oxidizing one μmol of NADPH/min at 37°C. No enzyme activity was detected when freshly prepared glucose was used as substrate. However, when the glucose solution was allowed to stand at 4°C for 3–4 days under sterile conditions, substantial enzyme activity was detected. Therefore, in all of the studies, aged α-D-glucose was used. This is not a unique phenomenon; Beutler and Matsumoto (16) demonstrated that galactose solution aged for 3–4 days at 4°C is a much better

Abbreviation: Glc-6-*P*, glucose 6-phosphate.

substrate for galactokinase. Concentrations of DL-glyceraldehyde and α-D-glucose exceeding 15 mM and 8 mM, respectively, significantly inhibited the activity of the native (unactivated) aldose reductase, whereas activated enzyme was not inhibited up to 50 mM glyceraldehyde and 30 mM glucose. With β-D-glucose solution aged 3–4 days, the enzyme activity was approximately 30% of that observed with α-D-glucose, whereas with freshly prepared β-D-glucose, no enzyme activity was detected. However, when the β-D-glucose solution was aged for more than 7 days at 4°C, it was as good a substrate as was aged α-D-glucose.

Sorbitol Determination. Sorbitol formed upon the reduction of glucose by DE-52-purified enzyme was quantitated fluorometrically by using sorbitol dehydrogenase (17, 18) and also by HPLC. In a 1.0-ml NADPH recycling system (wherever specified), 50 mM potassium phosphate (pH 6.5) was incubated for 90 min at 37°C with 5 mM 2-mercaptoethanol, 0.6 mM Glc-6-*P*, 100 milliunits of Glc-6-*P* dehydrogenase, 5 mM glucose containing 0.125 μCi of [1-^{14}C]glucose (specific activity, 8.2 mCi/mmol; 1 Ci = 37 GBq), and DE-52-purified erythrocyte aldose reductase. The reaction was stopped with 0.2 ml of 1 M HCl and brought to pH 7.0 by addition of 1 M NaOH. Samples were centrifuged at 2,000 × *g* for 10 min, and aliquots of supernatant were used for sorbitol determination by HPLC and fluorometry.

In the fluorometric method, sorbitol was quantitated by following the reduction of NAD by sorbitol dehydrogenase (17, 18) using a Turner model 111 fluorometer. In the HPLC method, 10–50 μl of the supernatant was injected onto a carbohydrate column (Bio-Rad 300 × 7.8 mm Aminex HPX-87P), equilibrated with water, flow rate 1 ml/min, at 76°C. Fractions of 0.5 ml were collected and the radioactivity was determined in Bray's solution (19) using a Beckman liquid scintillation system (model LS-230). [1-^{14}C]Glucose and [1-^{14}C]sorbitol were used as standards.

A distinct separation of glucose and sorbitol was achieved by HPLC (Fig. 1). Approximately 17% of the total glucose was reduced to sorbitol. Similar results were also obtained by the fluorometric method. Therefore, in subsequent determinations, fluorometry was used.

In a regular enzyme determination system, the rate of NADPH oxidation as well as the rate of sorbitol formation was also studied over a period of 25 min. The complete reaction mixture containing 5 mM glucose with and without Li_2SO_4 was incubated at 37°C with the appropriate amount of aldose reductase, and oxidation of NADPH was followed spectrophotometrically. Aliquots were withdrawn at different time intervals for the quantitation of sorbitol by the fluorometric method.

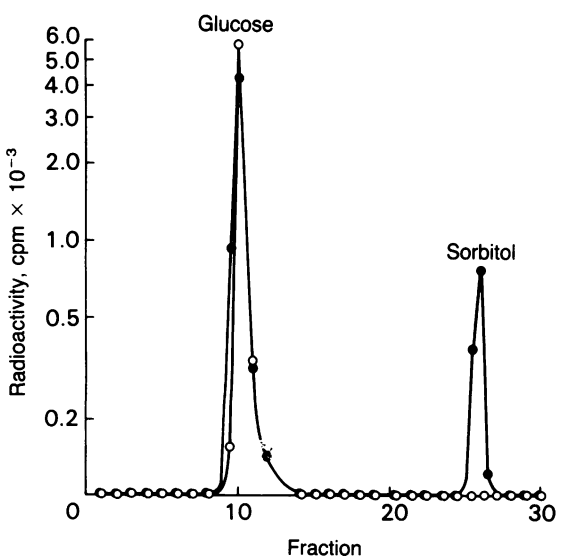

FIG. 1. Quantitation of sorbitol by HPLC. [1-^{14}C]Glucose was incubated in the NADPH-recycling system with human erythrocyte aldose reductase, and glucose and sorbitol were separated as described in the text. ●, Complete system; ○, without aldose reductase (control).

Activation of Aldose Reductase. Conditions for the activation of erythrocyte aldose reductase were optimized with glucose, Glc-6-*P*, and NADPH individually and together. Incubation of the enzyme with 10 μM each of Glc-6-*P*, NADPH, and glucose for 20 min at 25°C resulted in the maximum activation of the enzyme, which was unaffected by dialysis against buffer A. The dialyzed enzyme was designated "activated enzyme" and used for further studies. The enzyme that was not preincubated with activators was designated "unactivated" or "native enzyme."

RESULTS AND DISCUSSION

Effect of Glucose and Its Metabolites on the Erythrocyte Aldose Reductase. A significant increase in aldose reductase activity in the tissues of diabetic subjects and experimental animals has been reported (20–22). In addition, incubation of human erythrocytes with various concentrations of glucose (30–50 mM) resulted in the accumulation of substantial amounts of sorbitol in the erythrocytes (10, 11). These results indicate that glucose or one of its metabolites may be responsible for the increase in the enzyme activity, especially because erythrocytes cannot synthesize the enzyme *de novo*. One of the mechanisms of enzyme activation could be glucosylation of the enzyme protein.

In order to study the effect of glucose and its metabolites on erythrocyte aldose reductase, partially purified enzyme by DE-52 column chromatography was used. As described earlier (14), DE-52 column chromatography completely separated aldose reductase and aldehyde reductase II of erythrocytes. Differentiation of aldose reductase from aldehyde reductase II was based upon substrate specificity, cofactor requirements, and immunological properties (14). Aldose reductase can catalyze the reduction of glucose to sorbitol, uses NADPH as well as NADH as cofactors, and is precipitated by the antibodies raised against lens aldose reductase. Aldehyde reductase II does not utilize glucose as a substrate, exhibits an essential requirement for NADPH, and is precipitated with antiserum against placenta aldehyde reductase II but not with anti-lens aldose reductase antiserum.

Incubation of aldose reductase with 10 μM each of NADPH, Glc-6-*P*, and glucose at 25°C for 20 min resulted in approximately a 7-fold increase in the enzyme activity (Table 1). The activation of aldose reductase was comparatively less (4- to 6-fold) when outdated blood from the blood bank was used for the enzyme purification. Incubation of the enzyme with glucose alone did not activate the enzyme, whereas the incubation with Glc-6-*P* alone activated the enzyme approximately 3-fold. The enzyme remained activated even after thorough dialysis against buffer A or subjection to DE-52 column chromatography.

In order to study whether the activated enzyme forms proportionate amounts of sorbitol when glucose is used as substrate, the formation of sorbitol was followed (Fig. 2). Equal amounts of the native enzyme, purified by using outdated blood, was taken in two test tubes. In one tube the enzyme was incubated with 10 μM each of Glc-6-*P*, NADPH, and glucose for 20 min at 25°C, with the other tube serving as control. In both tubes, the sorbitol formation was determined in a 1-ml system containing 5 mM glucose, 50 mM potassium phosphate (pH 6.0), 5 mM 2-mercaptoethanol, and 0.1 mM NADPH. The tubes were incubated at 37°C, and aliquots were withdrawn at different time intervals for sorbitol deter-

Table 1. Activation of partially purified human erythrocyte aldose reductase

Activation conditions	Enzyme activity with different substrates, milliunits/ml	
	Glucose	Glyceraldehyde
Controls*		
NADPH added last	0.4 ± 1.0	6.6 ± 0.7
NADPH added first	3.5 ± 0.1	14.8 ± 1.4
Additions†		
Glc-6-*P*	7.3 ± 2.0	36.4 ± 3.4
Glc-6-*P* + NADPH	11.4 ± 2.3	52.9 ± 3.1
Glc-6-*P* + glucose	8.6 ± 1.9	25.5 ± 1.9
Glc-6-*P* + glucose + NADPH	23.4 ± 3.2	110.0 ± 5.3
Glc-6-*P* + glyceraldehyde + NADPH	20.1, 13.7	93.4, 55.2

Results represent mean ± SD of six experiments.

*In all the enzyme activity determinations except where mentioned otherwise, NADPH was added first in the reaction mixture and the substrate last.

†Partially purified erythrocyte enzyme (DE-52 step) was incubated with additions at 10 μM each for 20 min at 25°C and dialyzed 16–18 hr against 1 liter of buffer A at 4°C. After dialysis, all of the samples were adjusted to a fixed volume. The control samples were incubated without the additions and processed as the experimental samples. All of the dialyzed samples had comparable soluble protein concentrations. The enzyme activity was determined by using glucose or glyceraldehyde as substrate.

mination as described above. NADPH oxidation was also separately followed spectrophotometrically with the same system. Incubation of the native enzyme with the activation system resulted in approximately a 4-fold increase in the enzyme activity based upon the NADPH oxidation. The activated enzyme formed approximately 4-fold more sorbitol as compared with the native (unactivated) enzyme. In addition, the formation of sorbitol or NADPH oxidation was not affected by the presence or absence of Li_2SO_4 in the reaction mixture, although Li_2SO_4 has been reported to be required for the expression of aldose reductase activity by homogenous preparations from human erythrocytes and other tissues (15, 23–25).

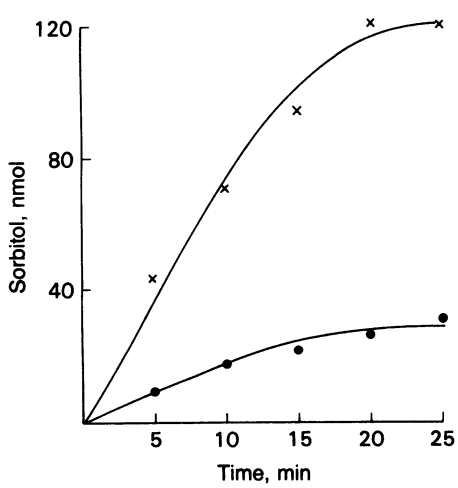

FIG. 2. Formation of sorbitol by activated (×) and unactivated (●) human erythrocyte aldose reductase. Partially purified aldose reductase was divided into two aliquots. One aliquot was incubated with 10 μM each of Glc-6-*P*, glucose, and NADPH for 20 min at 25°C, and the other aliquot was incubated without these additions. With glucose as substrate, the enzyme activity in both samples was determined by following NADPH oxidation and sorbitol formation. Sorbitol was quantitated fluorometrically by using sorbitol dehydrogenase as described in the text. The enzyme activity determined by either NADPH oxidation or sorbitol formation was linear for approximately 15 min.

The formation of sorbitol by activated and unactivated enzyme was also studied by following the competition between glyceraldehyde and glucose for the substrate binding site on aldose reductase. Various concentrations of glyceraldehyde (0–10 mM) were added to the NADPH recycling system for aldose reductase activity determination containing a fixed amount of glucose (5 mM). Samples were incubated at 37°C for 15 min, and the amount of sorbitol formed was determined fluorometrically. The results shown in Fig. 3 indicate that glyceraldehyde effectively competes with glucose for the binding center on both activated and unactivated aldose reductase. Only 0.2 mM glyceraldehyde was required for approximately a 50% decrease in the reduction of glucose because the K_m of aldose reductase for glyceraldehyde is almost 1/10th that for glucose.

Since Glc-6-*P* activated aldose reductase, the effect of various other phosphorylated intermediates of the glycolytic pathway, such as fructose 6-phosphate, fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, glycerate 1,3-bisphosphate, glycerate 2,3-bisphosphate, glycerate 3-phosphate, phospho*enol*pyruvate, AMP, ADP, and ATP on the activated and unactivated enzyme was studied. Both the activated and unactivated erythrocyte aldose reductase were incubated with 15 μM phosphorylated intermediates for 4 min at 37°C, and the enzyme activity was determined as described above. The unactivated enzyme was completely inhibited by glycerate 3-phosphate, glycerate 1,3-bisphosphate, glycerate 2,3-bisphosphate, and ADP, whereas these inhibitors had no effect on the activated enzyme (Table 2). The inhibition of the unactivated enzyme was not reversible even after a thorough dialysis against buffer A. None of the other intermediates had any significant effect on the activated or unactivated enzymes. Physiological concentrations of glycerate 3-phosphate (0.045 mM), glycerate 2,3-bisphosphate (4.2 mM), and ADP (0.22 mM) (26), besides completely inhibiting the unactivated aldose reductase, inhibited the activated enzyme also by 30–40%. However, significant amounts of these phosphorylated compounds may not exist *in vivo* in free form. Physiological concentrations of other phosphorylated intermediates had no effect on the activated or the unactivated enzyme.

Kinetic Properties of the Unactivated and Activated Aldose Reductase. The unactivated enzyme exhibited a biphasic curve with glucose (K_m = 9 and 0.9 mM) and with glyceraldehyde (K_m = 1.1 and 0.14 mM) (Fig. 4). The activated enzyme, on the other hand, exhibited monophasic kinetics with glucose (K_m = 0.68 mM) and with glyceraldehyde (K_m = 0.096 mM). These studies indicate that the native enzyme preparation probably contained a small amount of

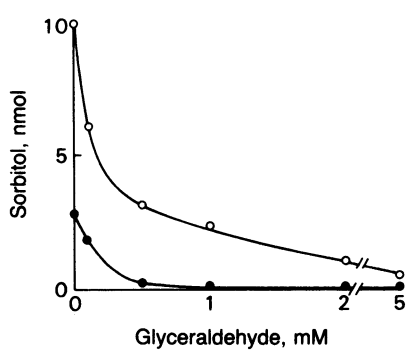

FIG. 3. Competition between glucose and glyceraldehyde for substrate binding site of activated (○) and unactivated (●) human erythrocyte aldose reductase.

Table 2. Effect of phosphorylated intermediates of glycolytic pathway on human erythrocyte unactivated and activated aldose reductase

Addition (15 μM)	Enzyme activity remaining, % of control	
	Unactivated enzyme	Activated enzyme
None	100	100
AMP	100	100
ADP	22	99
ATP	90	96
Glc-1-*P*	100	98
Glc-6-*P*	130	105
Fru-6-*P*	110	103
Fru-1,6-P_2	108	101
Gra-3-*P*	123	104
DHA-*P*	84	100
Glycerate 1,3-P_2	0	97
Glycerate 2,3-P_2	0	92
Glycerate 3-*P*	3	85
P-*e*Prv	88	96
Prv	93	98

Fru, fructose; Fru-1,6-P_2, fructose 1,6-bisphosphate; Gra-3-*P*, glyceraldehyde 3-phosphate; DHA-*P*, dihydroxyacetone phosphate; glycerate 1,3-P_2 and 2,3-P_2, glycerate 1,3-bisphosphate and 2,3-bisphosphate; Prv, pyruvate; *P*-*e*Prv, phospho*enol*pyruvate.

activated enzyme. Since the K_m of the activated enzyme for glucose is about 1/10th that of the unactivated enzyme and the V_{max} of the activated enzyme is much higher than that of the unactivated enzyme, the activated enzyme should more efficiently reduce glucose to sorbitol. As suggested by Inagaki *et al.* (27), if open-chain aldoglucose (constituting a minor percentage of D-glucose at equilibrium) is the true substrate of aldose reductase, the actual K_m for glucose will be still lower.

Effect of Aldose Reductase Inhibitors on the Activity of Unactivated and Activated Enzyme. The activated enzyme was less susceptible than the unactivated enzyme to inhibition by aldose reductase inhibitors such as sorbinil, alrestatin, and quercetrin. The results of sorbinil studies are given in Fig. 5. With glucose as substrate, the unactivated

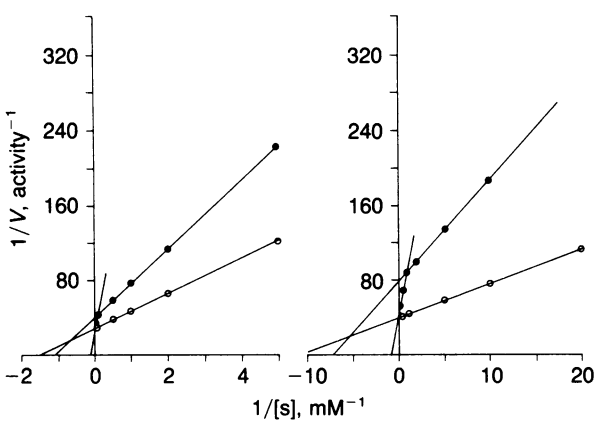

FIG. 4. Lineweaver–Burk plots of the activated (○) and unactivated (●) forms of human erythrocyte aldose reductase with glyceraldehyde (*Right*) and glucose (*Left*) as substrates. Enzyme activity was determined as described in the text with NADPH being added prior to the addition of substrate. Activation of aldose reductase was performed by incubating the enzyme with 10 μM each of Glc-6-*P*, NADPH, and glucose for 20 min at 25°C, followed by overnight dialysis against 1000 vol of buffer A. The amount of activated enzyme used in these studies was about 1/10th that of unactivated enzyme, so that the determined enzyme activities were almost equal. [s], Substrate concentration; *V*, velocity, units/ml.

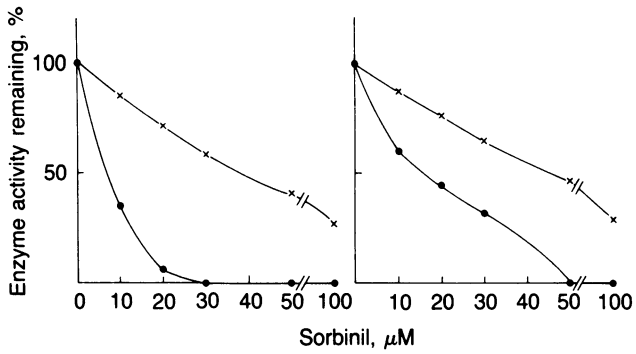

FIG. 5. The effect of various concentrations of sorbinil on the activity of the activated (×) and unactivated (●) forms of human erythrocyte aldose reductase with glyceraldehyde (*Right*) and glucose (*Left*) as substrates. The enzyme was activated as described in Fig. 4.

form of the enzyme was almost completely inhibited by 20 μM sorbinil, whereas the activated enzyme was inhibited by only 40%. With glyceraldehyde as substrate, the unactivated enzyme was completely inhibited by 50 μM sorbinil, whereas activated enzyme was inhibited by only 70% even with 100 μM sorbinil. Similar results were also obtained when alrestatin and quercetrin were used as inhibitors.

Formation of a significant amount of sorbitol in the erythrocytes of diabetic subjects having high blood glucose levels (10, 28) would indicate that a significant portion of the erythrocyte aldose reductase remains activated. We have indeed preliminary results that suggest that whenever blood glucose concentration in diabetic subjects is more than 15 mM, all of the erythrocyte aldose reductase is in the activated form (29). This enzyme exhibits properties similar to the *in vitro* activated erythrocyte aldose reductase. This would indicate that much higher concentrations of aldose reductase inhibitors will be required to completely inhibit erythrocyte aldose reductase in diabetic subjects. In fact, in clinical trials, the concentration of plasma sorbinil and alrestatin had to be maintained at approximately 50 μM (12) and 900 μM (13), respectively, to significantly lower the sorbitol concentration in erythrocytes of diabetic subjects and experimental animals, whereas approximately 20 μM sorbinil and 100 μM alrestatin almost completely inhibit the enzyme activity *in vitro* (30).

Activation of aldose reductase is not unique to erythrocytes. The enzyme of other human tissues such as lens, brain, aorta, and muscle also exists in the activated and unactivated forms and the unactivated form of aldose reductase can be activated to various degrees under conditions used for the activation of erythrocyte enzyme (29, 31). If aldose reductase is involved in the etiology of the complications of diabetes such as cataractogenesis, neuropathy, nephropathy, and retinopathy, our demonstration of the phenomenon of aldose reductase activation would significantly help in understanding the pathophysiology of these complications.

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