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Purification and Some Properties of Aldose Reductase from Rabbit Lens[1)]

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Four aldose reductases, designated here as aldose reductases Ia, Ib, IIa and IIb, have been purified to homogeneity from rabbit lens by a combination of several procedures such as ammonium sulfate precipitation, gel filtration, dye-affinity chromatography and chromatofocusing. The molecular weights of the four aldose reductases were estimated to be 33000 by Sephadex G-100 gel filtration, and to be 37000 by SDS-polyacrylamide gel electrophoresis. These enzymes had an identical pH optimum of 5.6. Substrate specificity studies showed that the four enzymes were capable of reducing various aldehydes and aldoses. However, D-hexoses (D-glucose and D-galactose) were poor substrates for aldose reductases IIa and IIb. Aldose reductases Ia and Ib used both reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as coenzymes, but aldose reductases IIa and IIb used NADPH specifically. Very high substrate concentrations were required to demonstrate the reaction in the reverse direction with these aldose reductases. Aldose reductases Ia and Ib were activated by sulfate ion, but aldose reductases IIa and IIb were not, and they were all inhibited remarkably by phenobarbital (1 mM). On the basis of the above results, aldose reductases Ia and Ib could be classified as aldose reductase (alditol: $NADP^+$ oxidoreductase, EC 1.1.1.21).

Keywords——purification; aldose reductase; rabbit lens; isozyme; substrate specificity; activation; inhibition; molecular weight

Aldose reductase [EC 1.1.1.21] is an enzyme which catalyzes the reduction of a variety of sugars to the corresponding sugar alcohols. Since Hers[2)] demonstrated that placenta and seminal vesicles contain aldose reductase, the enzyme has been found widely in a variety of mammalian[3–12)] and microbial[13,14)] sources. However, its metabolic significance in many of these cases is not yet definitely understood. In recent years, interest has been generated in aldose reductase because of its role in the pathogenesis of diabetic complications, including cataract, neuropathy and retinopathy. The presence of aldose reductase in the lens was first demonstrated by van Heyningen,[3)] and it has been suggested that the lens aldose reductase plays a leading role in the etiology of diabetic cataract. In brief, the presence of aldose reductase in the diabetic lens, combined with a high glucose level, results in a high intracellular sorbitol level and a consequent uptake of water which may lead to opacification of the lens, namely diabetic cataract. Although a number of aldose reductases have been found in mammalian tissues and their properties have been described,[3–12)] the properties of highly purified aldose reductase from lens are not known in detail, except in the case of calf lens aldose reductase, which has been investigated in detail by Sheaff and Doughty.[15)]

The purpose of this paper is to describe the isolation and some properties of aldose reductase from rabbit lens.

Experimental

Materials——Glycolaldehyde, D- and L-glyceraldehyde, D-erythrose and D-glucuronic acid (sodium salt) were purchased from Aldrich Chemical Co., and other aldehydes and aldoses were obtained from Wako Pure Chemical

Industries Ltd. Reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and $NADP^+$ were purchased from Sigma Chemicals. Mātrex gel orange A was obtained from Amicon Co. Polybuffer 74 and polybuffer exchanger 94 were purchased from Pharmacia Fine Chemicals. Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories. Lenses were removed from the eyes of rabbits of the Japanese White strain weighing 2—3 kg.

Standard Assay of Aldose Reductase——Aldose reductase activity was determined at 25 °C by measuring the decrease in absorption of NADPH at 340 nm on a Union High-Sens SM-401 spectrophotometer equipped with a National X-Y recorder. The assay mixture contained 100 mM sodium phosphate buffer (pH 6.2), 10 mM DL-glyceraldehyde, 0.15 mM NADPH and an appropriate amount of the enzyme in a final volume of 3.0 ml. The reaction was initiated by adding the enzyme, and the decrease of absorption at 340 nm was followed for 200 s. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per min under the conditions described here.

Polyacrylamide Gel Electrophoresis——Electrophoresis was carried out for 4 h in 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS) by the method of Shapiro *et al.*,[16] as described in detail by Weber and Osborn,[17] at a constant current of 8 mA per tube. After electrophoresis, the gel was stained for 15 h in 0.25% Coomassie brilliant blue solution. To estimate the molecular weight, aldose reductases and standard proteins such as cytochrome c (mol. wt. 11700), γ-globulin (L-chain, mol. wt. 23000; H-chain, mol. wt. 50000), ovalbumin (mol. wt. 43000) and bovine serum albumin (mol. wt. 68000) were incubated at 37 °C for 2 h in the presence of 1% SDS and 1% β-mercaptoethanol, and were subjected to electrophoresis at 8 mA per tube for 4 h.

Estimation of Molecular Weight by Gel Filtration——Gel filtration was performed on a Sephadex G-100 column (1.6 × 60 cm) according to the method of Andrews,[18] using proteins of known molecular weight, such as cytochrome c (mol. wt. 11700), myoglobin (mol. wt. 18000), chymotrypsinogen A (mol. wt. 25000), ovalbumin (mol. wt. 43000) and bovine serum albumin (mol. wt. 68000). The void volume of the column was determined by measuring the elution volume of blue dextran. Elution was performed with 0.05 M sodium phosphate buffer (pH 6.8) containing 0.1 M KCl and 2 mM dithiothreitol at a flow rate of 12 ml per h.

Protein Determination——The concentration of protein was determined with the Bio-Rad protein assay kit or by measuring the absorbance at 280 nm.

Purification of Aldose Reductase from Rabbit Lens——All operations were carried out at 4 °C in the presence of 2 mM dithiothreitol.

Step 1. Extraction: Twenty rabbit lenses (9.4 g) were homogenized in 35 ml of 20 mM sodium phosphate buffer (pH 6.8) containing 2 mM dithiothreitol (designated as Buffer A) with a Potter–Elvehjem homogenizer, and centrifuged at 41000 × *g* for 45 min to remove insoluble material.

Step 2. Ammonium Sulfate Fractionation: The supernatant obtained at step 1 was fractionated with solid ammonium sulfate. The precipitate recovered between 25 and 55% saturation was dissolved in 25 ml of buffer A, and dialyzed overnight against the same buffer to remove a great deal of the salt.

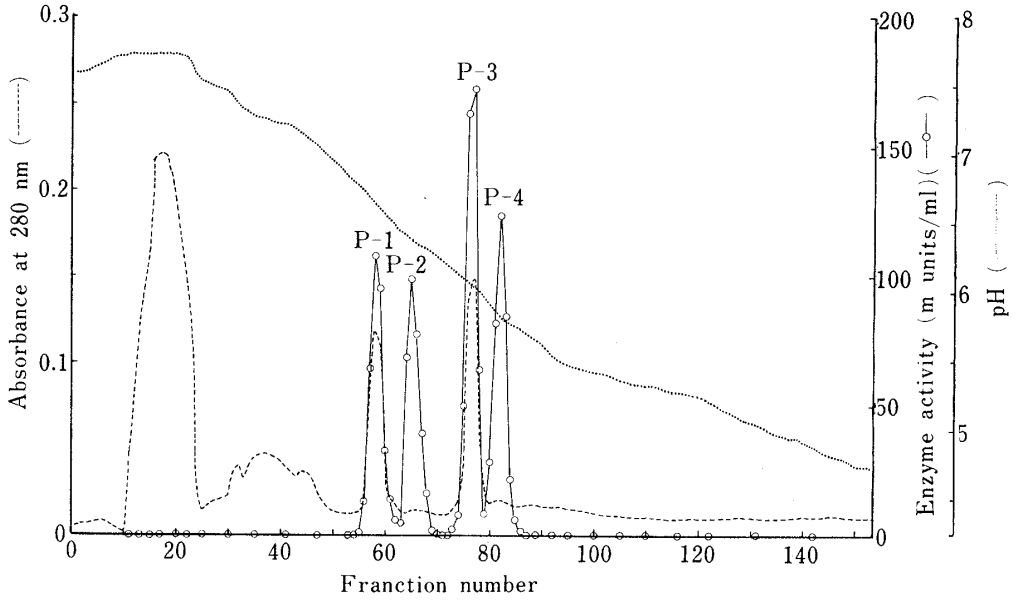


Fig. 1. Chromatofocusing of Rabbit Lens Aldose Reductase

For details of the procedure, see the text. —○—, aldose reductase activity; ----, absorbance at 280 nm; ------, pH.

Step 3. Sephadex G-200 Gel Filtration: The dialyzed enzyme preparation was applied to a column (2 × 90 cm) of Sephadex G-200 equilibrated with Buffer A. The enzyme was eluted with the same buffer at a flow rate of 15 ml per h, and 5-ml fractions were collected.

Step 4. Mātrex Gel Orange A Column Chromatography: The enzyme fraction from Sephadex G-200 gel filtration was applied to a column (1.6 × 25 cm) of Mātrex gel orange A equilibrated with Buffer A, and washed with the same buffer. The enzyme was eluted with a linear concentration gradient of potassium chloride, formed from 200 ml of Buffer A in the mixing chamber and 200 ml of Buffer A containing 1 M potassium chloride in the reservoir, at a flow rate of 35 ml per h. Aldose reductase activity was observed in the fraction eluted with about 0.4 M potassium chloride. Fractions with activity were pooled and concentrated by ultrafiltration using a collodion bag.

Step 5. Sephadex G-100 Gel Filtration: The concentrated enzyme solution from the previous step was placed on a column (1.6 × 60 cm) of Sephadex G-100 equilibrated with 0.025 M imidazole–HCl buffer (pH 7.4) and eluted with the same buffer at a flow rate of 15 ml per h. The enzyme fractions obtained were pooled and concentrated by ultrafiltration using a collodion bag.

Step 6. Chromatofocusing: The enzyme was subjected to chromatofocusing on a column (0.9 × 50 cm) of polybuffer exchanger 94 equilibrated with 0.025 M imidazole–HCl buffer (pH 7.4). The elution was carried out with polybuffer 74 adjusted to pH 4.5 at a flow rate of 20 ml per h, and 2.5-ml fractions were collected. The result of the chromatofocusing is shown in Fig. 1.

Results

Purification of Aldose Reductase from Rabbit Lens

As shown in Fig. 1, chromatofocusing revealed the presence of four kinds of enzymes with reducing activity towards DL-glyceraldehyde. The fractions in the tubes numbered 57—59, 64—67, 75—78 and 80—84 were combined into four fractions, which were tentatively designated as P-1, P-2, P-3 and P-4, respectively. From results of this study, the enzymes in both P-1 and P-3 were identified as aldose reductase and were termed aldose reductases Ia and Ib, respectively. Both P-2 and P-4 were found to contain a reductase which was not necessarily identifiable as aldose reductase, though these enzymes had properties in common with the enzymes in P-1 and P-3. The provisional names aldose reductases IIa and IIb were given to the enzymes in P-2 and P-4, respectively.

The results obtained at successive steps in the purification procedure are summarized in Table I. The four aldose reductases from rabbit lens in P-1, P-2, P-3 and P-4 were purified approximately 780-, 3620-, 1120- and 4430-fold, and were recovered in about 7, 9, 14 and 12% yields, respectively.

The enzyme preparations obtained were each homogeneous when examined by SDS-polyacrylamide gel electrophoresis (Fig. 2).

TABLE I. Purification of Aldose Reductase from Rabbit Lens

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purification (Fold)	Recovery (%)
1. Extraction	5886	7244	1.23	1	100
2. 25—55% ammonium sulfate fraction	4868	7350	1.51	1.2	101.5
3. Sephadex G-200 eluate	1825	6625	3.63	3.0	91.5
4. Mātrex gel orange A eluate	11.55	3445	298	242	47.6
5. Sephadex G-100 eluate	3.49	3273	937	762	45.2
6. Chromatofocusing					
P-1	0.524	505	964	784	7.0
P-2	0.152	682	4458	3624	9.4
P-3	0.786	1082	1376	1119	14.9
P-4	0.164	893	5445	4427	12.3

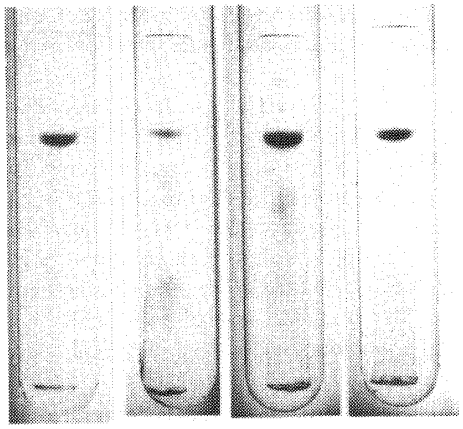


Fig. 2. SDS-polyacrylamide Gel Electrophoresis of Rabbit Lens Aldose Reductases

The enzymes were incubated at 37 °C for 2 h in the presence of 1% SDS and 1% β-mercaptoethanol, and were subjected to electrophoresis at 8 mA per tube for 4 h.

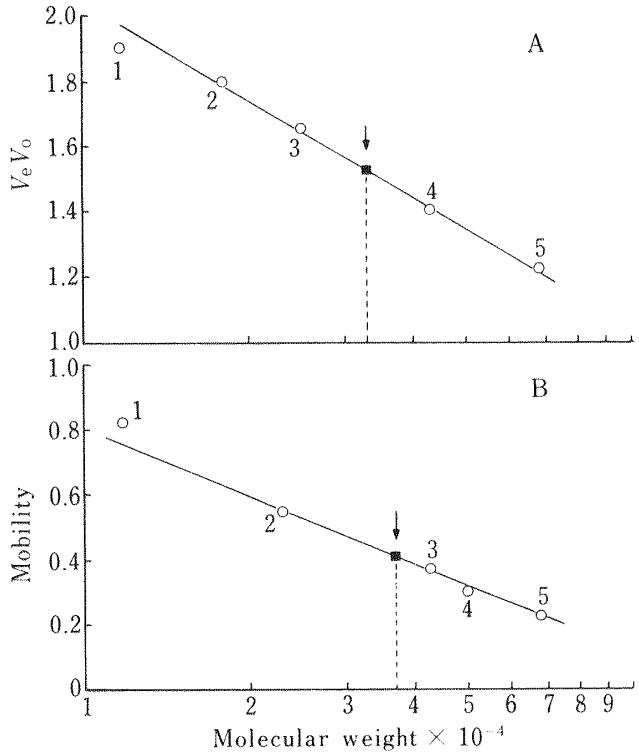


Fig. 3. Estimation of the Molecular Weight of Rabbit Lens Aldose Reductases

A) Gel filtration: The V_e/V_o ratios of standard proteins were plotted against molecular weight. The V_e/V_o ratios of aldose reductases are indicated by the arrow. The standard proteins used were: 1, cytochrome c; 2, myoglobin; 3, chymotrypsinogen A; 4, ovalbumin; 5, bovine serum albumin.

B) Electrophoresis in SDS-polyacrylamide gel: The mobilities of standard proteins were plotted against molecular weight. The mobilities of aldose reductases are indicated by the arrow. The standard proteins used were: 1, cytochrome c; 2, γ-globulin (L-chain); 3, ovalbumin; 4, γ-globulin (H-chain); 5, bovine serum albumin.

Estimation of Molecular Weight

The molecular weights of the four aldose reductases from rabbit lens were determined by gel filtration on a Sephadex G-100 column and by SDS-polyacrylamide gel electrophoresis. The elution volumes of all four enzymes were identical on a Sephadex G-100 column, and the approximate molecular weight of these enzymes was estimated to be 33000 (Fig. 3A). The mobilities of the four enzymes on SDS-polyacrylamide gel electrophoresis were also identical,

and the approximate molecular weight was estimated to be 37000 (Fig. 3B).

pH Optimum

The activities of the four aldose reductases in acetate, phosphate and Tris buffers at various pH values are shown in Fig. 4. The pH optima for the four enzymes measured with DL-glyceraldehyde as a substrate and NADPH as a coenzyme were identical, and were about pH 5.6. The pH-activity profile of aldose reductase Ia (P-1) had a strong similarity to that of aldose reductase Ib (P-3), and the pH-activity profiles of the two aldose reductase II's (P-2 and P-4) were also very similar. However, the pH-activity profiles of aldose reductase I's differed from those of aldose reductase II's at pH 7.2 or above. The relative activity at pH 7.6 of aldose reductase II's was about 50%, and that of aldose reductase I's was about 10%. The activities of the four aldose reductases in 0.1 M acetate buffer (pH 5.6) were less than a quarter of those in 0.1 M phosphate buffer (pH 5.6).

Substrate Specificity

Various aldoses and aldehydes were tested as substrates and the relative activities with those substrates are given in Table II. The range of substrates attacked by the enzymes from rabbit lens was fairly broad. All four aldose reductases were found to reduce short chain aliphatic aldehydes, an aromatic aldehyde and various aldoses, showing the greatest velocity with D- or DL-glyceraldehyde. Hexoses (D-glucose and D-galactose) were very poor substrates for aldose reductase IIa and IIb, and acetaldehyde was also a very poor substrate for all the enzymes. Aldose reductases Ia and Ib, and especially aldose reductases IIa and IIb, showed closely similar substrate specificities. When NADPH and NADH were compared as coenzymes for the reduction of DL-glyceraldehyde, it was found that although both NADPH and NADH served as coenzymes for all of the enzymes, the former was more effective than the latter. DL-Glyceraldehyde reduction could occur with NADH at about 40% of the rate with NADPH for aldose reductases Ia and Ib, but at only about 7% for aldose reductases IIa and IIb (Table II).

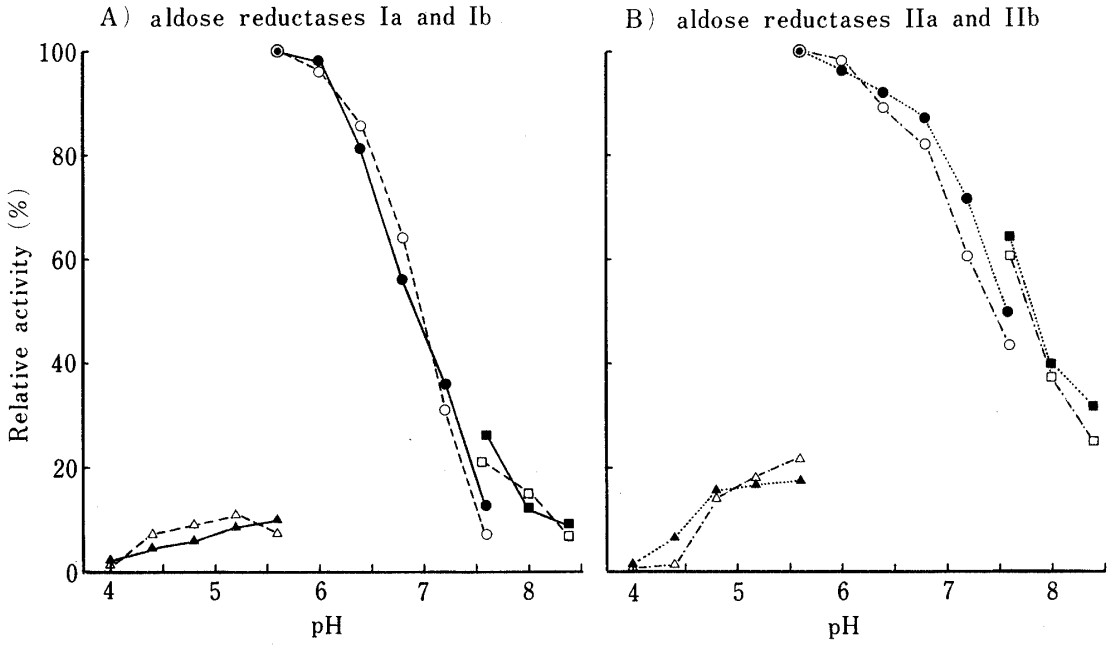


Fig. 4. pH Dependencies of Rabbit Lens Aldose Reductases

The standard assay was used except that the pH and buffer were varied. △ ▲, acetate buffer; ○ ●, phosphate buffer; □ ■, Tris-HCl buffer. ——, aldose reductase Ia; – – – –, aldose reductase Ib; ------, aldose reductase IIa; –·–·–·–, aldose reductase IIb.

Table II. Substrate and Coenzyme Specificity of Rabbit Lens Aldose Reductase Ia, Ib, IIa and IIb

Substrate	Concentration (mM)	Relative activity			
		Ia	Ib	IIa	IIb
DL-Glyceraldehyde	10	100	100	100	100
D-Glyceraldehyde	10	104	99	109	114
L-Glyceraldehyde	10	97	99	95	83
D-Erythrose	10	82	83	90	87
D-Xylose	10	48	38	14	16
D-Ribose	10	42	36	13	15
D-Glucose	10	14	11	1	1
D-Galactose	10	13	13	1	1
D-Glucuronolactone	10	85	58	17	24
D-Glucuronic acid	10	39	35	7	10
Glycolaldehyde	10	52	56	26	31
Acetaldehyde	1	2	4	1	1
Propionaldehyde	1	39	31	5	2
n-Butyraldehyde	1	86	92	42	41
Isobutyraldehyde	1	71	84	43	39
n-Valeraldehyde	1	48	47	72	66
Isovaleraldehyde	1	57	57	71	64
p-Nitrobenzaldehyde	0.2	79	62	62	60
NADPH	0.15	100	100	100	100
NADH	0.15	42	37	6	7

The standard assay was used except that 10 mM DL-glyceraldehyde or 0.15 mM NADPH was replaced by the substrate or coenzyme indicated. Relative activity is expressed as a percentage of the rate with DL-glyceraldehyde or NADPH.

Reverse Reaction

No reverse reaction of any of the four aldose reductases from rabbit lens could be detected in 0.1 M Tris-HCl buffer (pH 8.4) or in 0.1 M glycine–NaOH buffer (pH 9.6) with 0.22 mM $NADP^+$ and 10 mM glycerol. However, these enzymes could reduce $NADP^+$ under the following conditions: 0.1 M glycine–NaOH buffer (pH 9.6), 0.22 mM $NADP^+$ and 2 M glycerol or 1 M erythritol as a substrate. When the rates were compared with that found in the standard assay with 10 mM DL-glyceraldehyde and 0.15 mM NADPH, the relative rates for aldose reductases Ia, Ib, IIa and IIb with 2 M glycerol were 10, 5, 3 and 4%, respectively. Since the rate of $NADP^+$ reduction was not sufficient to permit accurate data to be obtained, this direction of the reaction was not investigated in detail.

Activation and Inhibition

As shown in Table III, the activities of aldose reductases Ia and Ib, like those from seminal vesicle, calf lens and human placental aldose reductases, increased 3.6 and 3.5 times, respectively, in the presence of 0.3 M ammonium sulfate. Similar activation was also observed in the presence of 0.3 M lithium sulfate or sodium sulfate. However, ammonium chloride, sodium chloride and magnesium chloride at 0.3 M concentration did not activate aldose reductase Ia or Ib. This finding suggested that the sulfate ion was the activating species. On the other hand, ammonium sulfate and lithium sulfate at 0.3 M concentration had no effect on the activities of aldose reductases IIa and IIb.

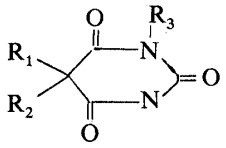
The inhibitory effects of barbiturates were determined for all four enzymes, and are shown in Table IV. The extents of inhibition of the four enzymes by these compounds were similar. Phenobarbital was the most potent inhibitor among various barbiturates tested; this

TABLE III. Effect of Salts on Rabbit Lens Aldose Reductase Ia, Ib, IIa and IIb Activities

Salt	Concentration (M)	Relative activity			
		Ia	Ib	IIa	IIb
None	—	100	100	100	100
$(NH_4)_2SO_4$	0.3	362	348	99	100
Li_2SO_4	0.3	355	357	101	98
Na_2SO_4	0.3	231	205	82	85
NH_4Cl	0.3	85	80	51	47
NaCl	0.3	70	74	50	51
$MgCl_2$	0.3	38	41	27	21

Relative activity is expressed as a percentage of the rate without salt.

TABLE IV. Inhibition of Aldose Reductase Ia, Ib, IIa and IIb from Rabbit Lens by Barbiturates



Compound	R_1	R_2	R_3	Inhibition (%)			
				Ia	Ib	IIa	IIb
Barbital	C_2H_5-	C_2H_5-	H	22	24	21	17
Pentobarbital	$CH_3CH_2CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}H-$	C_2H_5-	H	21	26	18	22
Amobarbital	$(CH_3)_2CHCH_2CH_2-$	C_2H_5-	H	13	19	9	14
Cyclobarbital		C_2H_5-	H	44	49	40	41
Hexobarbital		CH_3-	CH_3-	0	0	0	1
Phenobarbital		C_2H_5-	H	73	76	70	71

All compounds tested were added to the assay mixture to give a final concentration of 1 mM. Inhibition is expressed as a percentage calculated by comparison with the uninhibited control.

compound diminished the activities of all four enzymes by about 70%. Barbital, pentobarbital and amobarbital slightly inhibited (about 20%), and hexobarbital did not affect the activities of these enzymes.

Discussion

Aldose reductase has so far been found in mammalian tissues such as calf,[4-7] human,[8-10] rat,[11] pig[12] and sheep,[2] and in microorganisms such as *Rhodotorula*[13] and *Pichia quercuum*.[14] The present paper demonstrates that rabbit lens contains four kinds of aldose reductases. These enzymes from rabbit lens were separated by chromatofocusing, and the isolated enzymes were each proved to be homogeneous by SDS-polyacrylamide gel electrophoresis.

The molecular weights of the four aldose reductases from rabbit lens were identical, and all four enzymes were found to have a molecular weight of 33000—37000. These values are

similar to the values of 29000 to 40000 reported for aldose reductases from various sources.[6-8,12,15]

The pH optima of all four aldose reductases from rabbit lens were identical, and were pH 5.6. This value is the same as for the aldose reductase from calf lens.[4] Human placental aldose reductase has a broad pH optimum between pH 5.8 and 6.7,[8] and the optimum pH for the enzymes from human[9] and porcine[12] brain are pH 6.6 and 5.0, respectively. The substrate specificities of the four aldose reductases from rabbit lens were fairly broad. With regard to the broad substrate specificity, the rabbit lens aldose reductases generally resembled the enzymes from other sources.[2a,4,6-9,12] However, among the rabbit lens enzymes, aldose reductase I's (Ia and Ib) differ from aldose reductase II's (IIa and IIb) in their activities with D-hexoses (D-glucose and D-galactose) and in their coenzyme requirement. Although the activity of aldose reductase I's for glucose was about 10% of that for DL-glyceraldehyde, that of aldose reductase II's for glucose was extremely low. Further, the rate of NADH oxidation by aldose reductase I's was about 40% of that of NADPH oxidation, whereas aldose reductase II's were specific for NADPH. The substrate specificity of aldose reductase I's was quite similar to those of the human placental,[8] sheep seminal vesicle[2a] and calf lens[4] enzymes. Both D- and L-enantiomers of glyceraldehyde could act as substrates for all four aldose reductases from rabbit lens. Similar results have been reported for the enzymes from calf liver,[6] rabbit skeletal muscle[19] and *Rhodotorula*.[13] It has been reported that high substrate concentrations are required to demonstrate the reverse reaction of aldose reductase from calf lens,[4] calf liver[6] and *Pichia quercuum*.[14] In the case of rabbit lens aldose reductases, all four enzymes catalyzed the $NADP^+$-dependent oxidation of 2 M glycerol and 1 M erythritol in 0.1 M glycine–NaOH buffer (pH 9.6), but these reactions proceeded at only about 3—10% of the reduction rate of 10 mM DL-glyceraldehyde at pH 6.2, and no oxidation of 10 mM glycerol was observed in the presence of $NADP^+$ at pH 9.6. Thus, the enzymes from rabbit lens would seem to function physiologically as aldose reductases rather than as polyol dehydrogenases.

O'Brien and Schofield have reported the inhibition of human brain aldose reductase by barbiturates.[9] The rabbit lens aldose reductases were also inhibited by these compounds. Because phenobarbital was the most potent inhibitor among barbiturates tested, an aromatic substituent on the C-5 position of barbiturate may enhance the inhibitory effect. On the other hand, the introduction of a substituent at the N-1 position may result in a significant decrease in inhibitory effect, since hexobarbital hardly inhibited the enzyme activities.

Hers found first that the sheep seminal vesicle aldose reductase was activated by sulfate ion,[2a] and this has been confirmed and extended to other aldose reductases from calf lens,[4] calf brain,[7] human placental[8] and porcine brain.[12] We also observed the activation of two enzymes in rabbit lens, aldose reductases Ia and Ib, by sulfate ion. In this respect, rabbit lens aldose reductase I's resemble the aldose reductases from other sources described above. However, rabbit lens aldose reductase II's were not activated by sulfate ion. Thus, rabbit lens aldose reductase II's differ from aldose reductase I's and aldose reductases from other sources in the effect of sulfate ion.

From the results in this paper, it seems likely that aldose reductases Ia and Ib are isozymes and can best be classified as aldose reductase (alditol: $NADP^+$ oxidoreductase, EC 1.1.1.21).

The accumulation of intracellular sorbitol can cause a hyperosmotic effect which results in cellular swelling.[20] In diabetic animals, aldose reductase, which catalyzes the reduction of glucose to sorbitol, has been shown to initiate cataract formation.[3,21-23] In a previous paper,[24] we have reported that sorbitol, which can arise from glucose, was accumulated in rabbit lens in the diabetic state. Upon comparing various enzymatic properties of aldose reductases from other sources, aldose reductases Ia and Ib rather than aldose reductase IIa and IIb seem more likely to be important in the accumulation of sorbitol in diabetic rabbit

lens, namely in the pathogenesis of diabetic cataract.

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