Biochemical Pharmacology, Vol. 46, No. 6, pp. 1043–1052, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

NADPH-DEPENDENT ENZYME-CATALYZED REDUCTION OF ALDOPHOSPHAMIDE, THE PIVOTAL METABOLITE OF CYCLOPHOSPHAMIDE

HEMANT K. PAREKH and NORMAN E. SLADEK*
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

(*Received* 4 *December* 1992; *accepted* 5 *May* 1993)

Abstract—One of the metabolites found in the urine of mammals given the prodrug cyclophosphamide is alcophosphamide, an alcohol. It is most probably generated from cyclophosphamide via aldophosphamide, an aldehyde which otherwise can directly give rise to phosphoramide mustard; the latter effects the cytotoxic action of cyclophosphamide and other oxazaphosphorines. It has already been demonstrated that horse liver alcohol dehydrogenase catalyzes the reduction of aldophosphamide to alcophosphamide. Herein, we report that aldose reductase and aldehyde reductase purified from human placenta also catalyze this reaction. The K_m values for aldose reductase- and aldehyde reductase-catalyzed reduction of aldophosphamide to alcophosphamide were 0.15 and 1.6 mM, respectively. Aldose reductase and aldehyde reductase accounted for 94 and 6%, respectively, of total placental pyridine nucleotide-dependent enzyme-catalyzed aldophosphamide (160 μM) reduction. Aldose reductase-catalyzed reduction of aldophosphamide appeared to be noncompetitively inhibited by sorbinil; the K_i value was 0.4 μM. The *in vivo* significance of these observations is uncertain but could be of some magnitude since alcophosphamide is known to be only weakly cytotoxic.

Cyclophosphamide and certain other oxazaphosphorines, e.g. mafosfamide and 4-hydroperoxycyclophosphamide, are therapeutically useful anticancer agents which, *per se*, are pharmacologically inert. Activation of these agents, as well as subsequent inactivation of the intermediary metabolites, is a complex process and has been the subject of many investigations [reviewed in Refs. 1 and 2]. Salient features of oxazaphosphorine metabolism are presented in Fig. 1.

Germane to the present investigation are the reports that alcophosphamide, a metabolite of cyclophosphamide albeit apparently a minor one [3–8], is only weakly cytotoxic [9, 10]. Formation of alcophosphamide, an alcohol, is likely to be from aldophosphamide, an aldehyde which otherwise can directly give rise to phosphoramide mustard; the latter effects the cytotoxic action of cyclophosphamide and other oxazaphosphorines. Reduction of aldophosphamide to alcophosphamide is almost certainly enzyme-catalyzed and reversible. Direct conversion of alcophosphamide to phosphoramide mustard is also a possibility [7]. Thus, formation of alcophosphamide can be viewed as an enzyme-catalyzed, reversible detoxification.

The identity of the enzyme(s) that catalyzes the reduction of aldophosphamide to alcophosphamide is essentially unknown. To date, only commercially available horse liver alcohol dehydrogenase is a demonstrated catalyst of this reaction [6]. There is no information as to the identify of the human enzyme(s) that might be involved. Candidates, because they catalyze the reduction of carbonyls and are somewhat substrate nonspecific, include each of the several isoforms of alcohol dehydrogenase, and a group of enzymes collectively termed pyridine nucleotide-dependent aldo-keto reductases, viz. aldehyde reductase (EC 1.1.1.2; also known as high K_m aldehyde reductase and L-hexonate dehydrogenase), aldose reductase (EC 1.1.1.21; also known as low K_m aldehyde reductase) and carbonyl reductase (EC 1.1.1.184; also known as prostaglandin 9-ketoreductase and daunorubicin-pH 6.0 reductase).

Aldehyde reductase and aldose reductase are abundantly present in human placenta [11–13]. Herein, we report on the relative efficacy and importance of these two enzymes in catalyzing the reduction of aldophosphamide to alcophosphamide.

MATERIALS AND METHODS

Materials. 4-Hydroperoxycyclophosphamide and alcophosphamide were provided by Dr. Jorge Pöhl, Asta-Werke AG, Bielefeld, F.R.G., and Dr. Kenneth K. Chan, Ohio State University, Columbus, OH, respectively. NADP, NADPH, DL-glyceraldehyde, D-glucuronic acid, D-glucose, D-xylose, bovine serum albumin (crystallized and lyophilized), sodium dodecyl sulfate (SDS†), dithiothreitol, Lubrol®, phenobarbital, sodium valproate, quercetin, Coomassie Brilliant Blue R-250, Sephacryl S-200, standard protein marker kits for gel permeation and SDS–polyacrylamide gel elec-

* Corresponding author: Dr. N. E. Sladek, Department of Pharmacology, University of Minnesota, 3-249 Millard Hall, 435 Delaware Street S.E., Minneapolis, MN 55455. Tel. (612) 625-0691; FAX (612) 625-8408.

† Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBE, polybuffer exchanger; pI, isoelectric point; and ALDH-1, human aldehyde dehydrogenase-1.

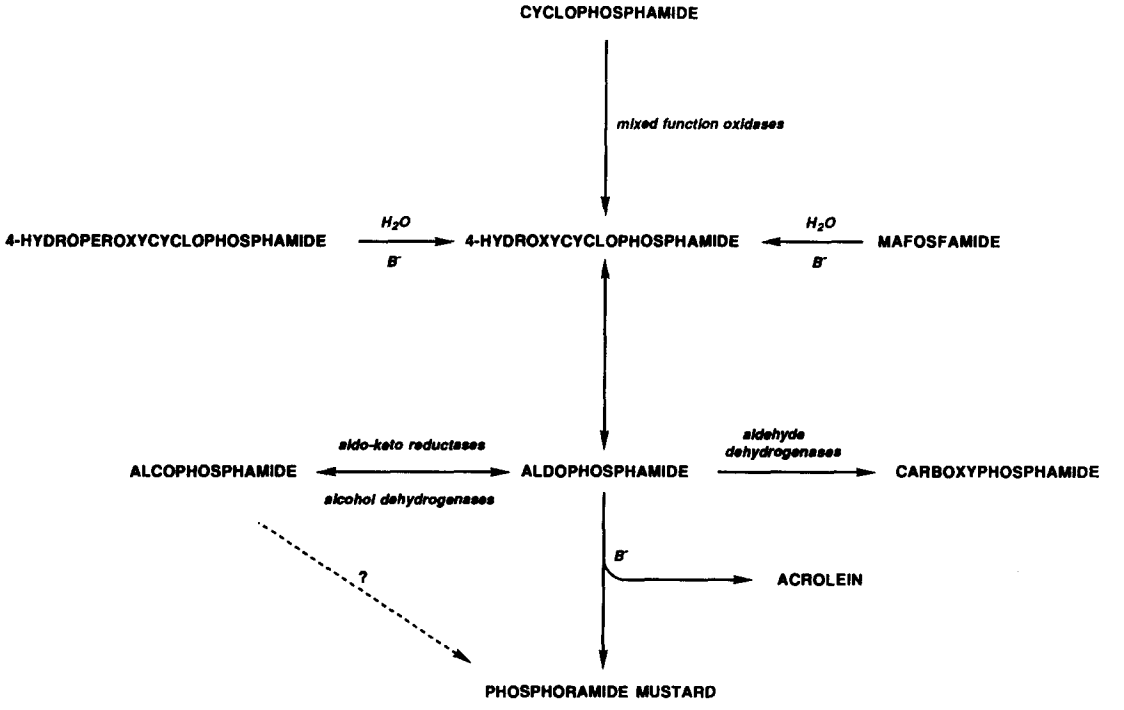

Fig. 1. Salient features of oxazaphosphorine metabolism [reviewed in Refs. 1 and 2]. The prodrugs cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines. Alternatively, aldophosphamide can be either oxidized to carboxyphosphamide or reduced to alcophosphamide. Putatively, catalysis of the latter is by one or more of the alcohol dehydrogenases and/or aldo-keto reductases. Carboxyphosphamide and alcophosphamide are, themselves, without cytotoxic activity. The former does not give rise to a cytotoxic metabolite. However, alcophosphamide apparently retains the potential to give rise to phosphoramide mustard [7].

trophoresis (PAGE), and goat anti-rabbit IgG conjugated to alkaline phosphatase were purchased from the Sigma Chemical Co., St. Louis, MO. Matrex Gel Orange A, Centriprep-10 concentrators and YM-10 ultrafiltration membranes were purchased from the Amicon Division, W. R. Grace & Co., Danvers, MA. Red Sepharose CL 6B, polybuffer exchanger (PBE) 94, polybuffer 74, PD-10 columns, Ampholine PAGplates (pH 3.5 to 9.5) and an isoelectric point (pI) marker kit were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ. Protein assay dye reagent concentrate, acrylamide and *N,N'*-methylene-bis-acrylamide were obtained from Bio-Rad Laboratories, Richmond, CA. Immobilon-PVDF membranes were purchased from the Millipore Corp., Bedford, MA. Non-fat dried milk was purchased from a local supplier.

Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide as described previously [14].

Two human placenta samples were obtained from the Tissue Procurement Facility, University of Alabama Comprehensive Cancer Center, Birmingham, AL, through the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. The supplier certified each sample to be nonpathological. Each of the two samples was kept at 0–4° and was delivered to us within 24 hr of donation. Aldo-keto reductase activity present in the first sample received was determined immediately upon arrival and after freezing at −70° for several weeks. Enzyme purification was from the second sample received; it had been stored at −70° for several weeks prior to the initiation of purification.

Rabbit antiserum to bovine lens aldose reductase and sorbinil (a spirohydantoin; *d*-6-fluorospiro-[chroman-4,4'-imidazolidine]-2',5'-dione; Pfizer) was provided by Dr. J. M. Petrash, Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO. Rabbit antiserum to human liver aldehyde reductase was provided by Dr. D. L. Vander Jagt, Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, NM.

Buffer A was 1 mM EDTA and 10 mM sodium phosphate in aqueous solution, pH 7.0. Buffer B was 0.1 mM dithiothreitol and 10 mM potassium phosphate in aqueous solution, pH 7.4. Buffer C was 25 mM imidazole-HCl in aqueous solution,

pH 7.4. Buffer D was 20 mM sodium phosphate in aqueous solution, pH 7.0. All buffers used for column chromatography were degassed prior to use.

Preparation of subcellular fractions. After removing membranous and connective tissues, placenta samples were homogenized in 10 vol. of Buffer A with the aid of a Waring blender (three 1-min bursts). After further homogenization in a Dounce homogenizer (loose fitting pestle; 3 strokes), the whole homogenate was centrifuged at 105,000 *g* and 4° for 60 min to generate a supernatant (soluble) and a pellet (particulate) fraction. The former was transferred with the aid of PD-10 columns into Buffer B supplemented with 0.1 mM NADP and saved for further use. The pellet fraction was solubilized by placing it in a 0.3% Lubrol® in Buffer A solution, and this preparation was centrifuged at 105,000 *g* and 4° for 60 min. The resulting supernatant (solubilized particulate) fraction was saved for further use.

Chromatographic resolution and purification of human placenta aldo-keto reductases. Resolution and purification of placenta aldehyde reductase and aldose reductase was achieved essentially as described by Vander Jagt *et al.* [13] except that column chromatography (Affinity Matrex Gel Orange A; 1 × 45 cm) rather than HPLC (Bio-Gel HPHT hydroxylapatite) was used for the final purification step, i.e. after Red Sepharose (2.5 × 20 cm) and PBE 94 (1 × 40 cm) column chromatography. Linear flow rates were 50, 20 and 30 mL/hr, respectively. All procedures were performed at 4°. The spectrophotometric assay described below was used to monitor aldo-keto reductase activity in eluate fractions. Elution of protein from chromatography columns was routinely monitored at 280 nm with an ISCO UA-5 absorbance monitor. The pH of selected eluate fractions was determined with a Corning glass pH electrode. Where indicated, the volume of pooled eluate fractions was reduced using an Amicon ultrafiltration stirred-cell apparatus fitted with a YM-10 membrane and pressurized under nitrogen, or using an Amicon Centriprep-10 concentrator. Transfer of enzyme fractions from one salt/buffer solution to another was accomplished with the aid of Pharmacia PD-10 gel filtration columns. Purified enzymes were transferred into a solution of 0.1 mM dithiothreitol and 10 mM sodium phosphate, pH 7.0, prior to immunoblot analysis and determination of molecular weight, pI values, Michaelis–Menten kinetic constants and sensitivity to putative inhibitors and activators.

Protein determination. The protein content of crude tissue fractions and selected column pools was determined by the Coomassie Brilliant Blue dye binding assay [15] using commercially available Bio-Rad Protein Assay reagent and bovine serum albumin as the standard.

Assay for aldo-keto reductase activity. Except as noted, the assay mixture (1 mL, pH 7.0) consisted of 0.16 mM NADPH, 100 mM sodium phosphate, the substrate of interest, and crude fraction or (semi)purified enzyme. The reaction was started by the addition of substrate, and the disappearance of NADPH at 37° was monitored at 340 nm with the aid of a Beckman DU-70 recording spectrophotometer. An assay mixture containing all of the components except the substrate served as the blank. Initial rates were determined in duplicate in all experiments. It was determined in preliminary experiments that incubation of alcophosphamide with NADPH and crude fraction or (semi)purified enzyme gave rise to a single aldophosphamide metabolite which, upon submission to thin-layer co-chromatography, migrated exactly as did authentic alcophosphamide. Enzyme activities were reduced by more than 90% when 0.16 mM NADH was substituted for NADPH.

Isoelectric focusing. Isoelectric focusing was as described by Dockman *et al.* [14] except that a constant power output of 20 W for 3000 V-hr was used. Coomassie Brilliant Blue R-250 (0.05%) was used to visualize proteins. A solution of water:methanol:acetic acid (6:3:1) was used to destain gels.

Molecular weight determinations. A Sephacryl S-200 gel filtration column equilibrated with an aqueous solution of 100 mM NaCl and 50 mM sodium phosphate, pH 7.0, was used to determine molecular weights under nondenaturing conditions. The SDS–PAGE method of Laemmli [16] was used to determine molecular weights under denaturing conditions.

Immunoblot analysis. Purified aldo-keto reductases were submitted to SDS–PAGE [16] and were then electrotransferred onto an Immobilon-PVDF transfer membrane as described by others [17] using a Bio-Rad semidry blotter. Immunodetection of aldose reductase and aldehyde reductase on the blotted membrane was as previously described [18] except that 5% (w/v) non-fat dried milk, rather than 3% gelatin, was used in the blocking solution.

Data analysis. Double-reciprocal plots of initial rates versus substrate concentrations were used to calculate all K_m and V_{max} values. Wilkinson weighted linear regression analysis [19] was used to fit lines to the double-reciprocal plot values. The K_i value for sorbinil was determined as described previously [14]; sorbinil concentrations were 0, 0.1, 0.25, 0.5 and 1 μM. Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight line functions.

RESULTS

Preliminary experiments established that soluble (105,000 *g* supernatant) fractions prepared from fresh human placenta contained one or more reduced pyridine nucleotide-dependent enzyme(s) capable of catalyzing the reduction of aldophosphamide as well as that of DL-glyceraldehyde and D-glucuronate, and that solubilized (105,000 *g* pellet) particulate fractions did not. It was also noted that storage of placental samples at −70° for several weeks did not result in a significant loss of reductase activity in subsequently prepared 105,000 *g* supernatant fractions.

A 105,000 *g* supernatant fraction prepared from a human placental sample that had been stored at −70° for several weeks was then passed through a series of chromatography columns in order to resolve, purify and identify individual reduced pyridine nucleotide-dependent enzymes capable of

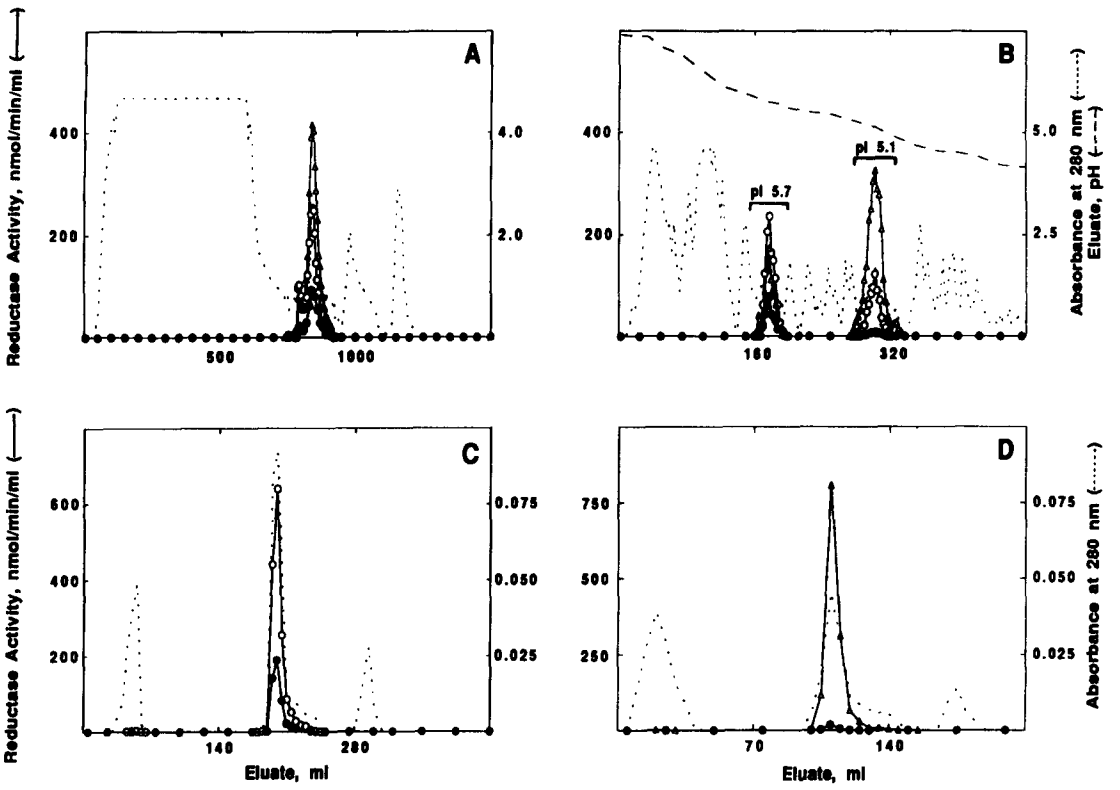

Fig. 2. Chromatographic separation and purification of human placenta aldo-keto reductases. (A) A 105,000 *g* supernatant fraction (7,569 mg protein in 110 mL of Buffer B supplemented with 0.1 mM NADP) obtained from 160 g of placenta was loaded onto a Red Sepharose column equilibrated with Buffer B containing 0.1 mM NADP. The loaded column was washed successively with 600 mL of 200 mM potassium phosphate, pH 7.4, 500 mL Buffer B containing 2 mM NADP and 0.5 M NaCl, and 400 mL Buffer B containing 1 M NaCl. (B) Reductase activity recovered from the Red Sepharose column was pooled (400 mL), concentrated 16-fold, and transferred into Buffer C supplemented with 0.1 mM NADP. This preparation (35 mL) was loaded onto a PBE 94 column that had been equilibrated with Buffer C. The column was developed with 11% polybuffer 74, pH 4.0, supplemented with 0.1 mM NADP. (C) Pooled pI 5.7 reductase activity (30 mL) from the PBE 94 column (Fig. 2B) was transferred into Buffer D supplemented with 0.1 mM NADP. This preparation (41 mL) was loaded onto a column of Matrex Gel Orange A affinity resin equilibrated with Buffer D. The loaded column was washed successively with 125 mL of Buffer D, 125 mL of Buffer D supplemented with 0.1 mM NADPH, and 125 mL Buffer D supplemented with 1 M NaCl. (D) Pooled pI 5.1 reductase activity (42 mL) from the PBE 94 column (Fig. 2B) was concentrated 7-fold and then transferred into Buffer D. This preparation (10 mL) was loaded onto a column of Matrex Gel Orange A affinity resin equilibrated with Buffer D. The loaded column was washed successively with 75 mL Buffer D, 75 mL Buffer D supplemented with 0.1 mM NADPH, and 40 mL Buffer D supplemented with 1 M NaCl. (A–D) Eluates were collected in 2.5- to 10-mL fractions. Substrates used to monitor reductase activity were 10 mM DL-glyceraldehyde (○), 10 mM D-glucuronic acid (△) and 0.16 mM aldophosphamide (●). Not all of the data obtained with these substrates is shown.

catalyzing the reduction of aldophosphamide (Fig. 2, Table 1). Two such enzymes, viz. a pI 5.7 reductase ultimately identified as aldose reductase, and a pI 5.1 reductase ultimately identified as aldehyde reductase, were resolved and purified. Identification of the pI 5.7 and 5.1 enzymes as aldose reductase and aldehyde reductase, respectively, was largely on the basis of molecular weight determinations, pI values, antibody recognition, and substrate preference, *vide infra*.

Molecular weights for the pI 5.7 and 5.1 enzymes as determined by Sephacryl S-200 gel permeation chromatography, i.e. under nondenaturing conditions, were 33,100 and 36,300, respectively (data not presented). Molecular weights determined under denaturing conditions, viz. SDS–PAGE, were nearly identical, viz. 35,000 and 39,000, respectively (Fig. 3), suggesting that both proteins are monomers. Consistent with the notion that each of these enzymes was purified to homogeneity were the observations that only one protein was observed in each of these experiments (Sephacryl S-200 gel permeation chromatography; SDS–PAGE) in the case of each enzyme.

Consistent with the pI values of 5.7 and 5.1 obtained by chromatofocusing (Fig. 2B), values of

Table 1. Purification of aldose and aldehyde reductases from human placenta*

Fraction	Total protein (mg)	Total activity (nmol/min)			Specific activity (nmol/min/mg protein)		
		Glyceraldehyde	Glucuronate	Aldophosphamide	Glyceraldehyde	Glucuronate	Aldophosphamide
Soluble (105,000 *g* Supernatant) fraction							
Total	7,569	19,608	29,355	5,461	2.6	3.9	0.7
Red Sepharose CL 6B							
Pooled reductase	82.4	26,910†	28,444	6,726†	327	345	81
PBE 94							
Pooled pI 5.7 reductase	7.3	14,368	7,007	4,395	1,968	960	602
Pooled pI 5.1 reductase	3.3	5,296	11,535	262	1,605	3,495	79
Matrex Gel Orange A							
Pooled pI 5.7 reductase	5.0	14,343	7,017	4,402	2,869	1,403	880
Pooled pI 5.1 reductase	0.94	5,530	12,512	266	5,883	13,311	283

* Purification was as described in the legend of Fig. 2 and the text. Glyceraldehyde, glucuronate and aldophosphamide concentrations were 10, 10 and 0.16 mM, respectively. Ultimately, the pI 5.7 and pI 5.1 enzymes were identified as aldose reductase and aldehyde reductase, respectively.

† The basis for the greater total enzyme activity in the pooled Red Sepharose eluate relative to that in the starting soluble (105,000 *g* supernatant) fraction is not apparent. Vander Jagt *et al.* [13] observed the same phenomenon and suggested that it could be due to the elimination of a substance, e.g. hemoglobin, that inhibits one or more of the reductases.

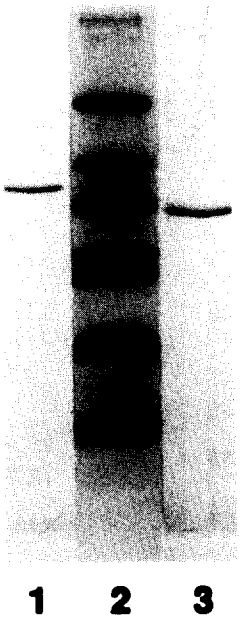

Fig. 3. Molecular weight determination of pI 5.7 and pI 5.1 reductases purified from human placenta. SDS–PAGE of pI 5.7 and pI 5.1 reductases purified from human placenta (lanes 3 and 1, respectively; 2 μg each) was on a discontinuous gel, viz. a 12% separating gel and a 4% stacking gel. Molecular weight standards in lane 2 were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.2 kDa). Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

5.2 to 5.75 (two major and two minor bands) and 5.1 (a single band), respectively, were obtained when the two enzymes were submitted to isoelectric focusing (Fig. 4).

Antiserum to human liver aldehyde reductase recognized the pI 5.1 enzyme (Fig. 5, lane 2) and displayed a weak cross-reactivity with the pI 5.7 enzyme (Fig. 5, lane 3). Previously, Vander Jagt *et al.* [13] reported that antiserum to human liver aldehyde reductase cross-reacted weakly with human placental aldose reductase. Antiserum to bovine lens aldose recognized the pI 5.7, but not the pI 5.1, enzyme (Fig. 5, lanes 5 and 4, respectively).

As judged by K_m and V_{max} values (Table 2), the pI 5.7 enzyme favored DL-glyceraldehyde over D-glucuronate as a substrate, and the pI 5.1 enzyme did just the opposite. Normal hyperbolic kinetics were observed in every case, i.e. regardless of the enzyme or substrate utilized. Substrate inhibition was not observed in any case.

The catalytic activity of the pI 5.1 enzyme was essentially unaffected by the presence of 0.4 M lithium sulfate or ammonium sulfate in the reaction mixture (data not presented). In contrast, the activity of the pI 5.7 enzyme was enhanced *ca.* 2-fold when

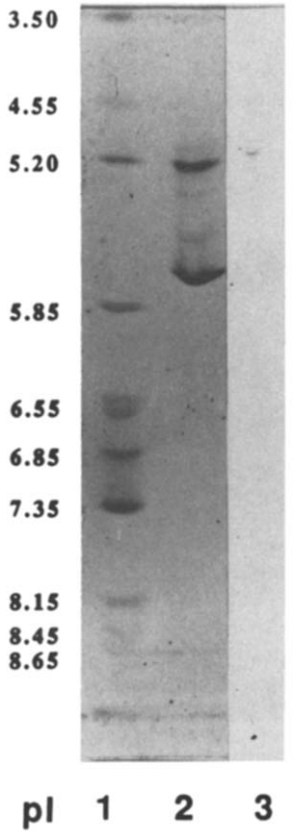

Fig. 4. Isoelectric focusing of the pI 5.7 (putatively, aldose reductase; lane 2) and pI 5.1 (putatively, aldehyde reductase; lane 3) enzymes purified from human placenta. Isoelectric focusing of the pI standards (lane 1) and the two enzymes (10 μg of each) was as described in Materials and Methods. Coomassie Brilliant Blue R-250 was used to visualize proteins.

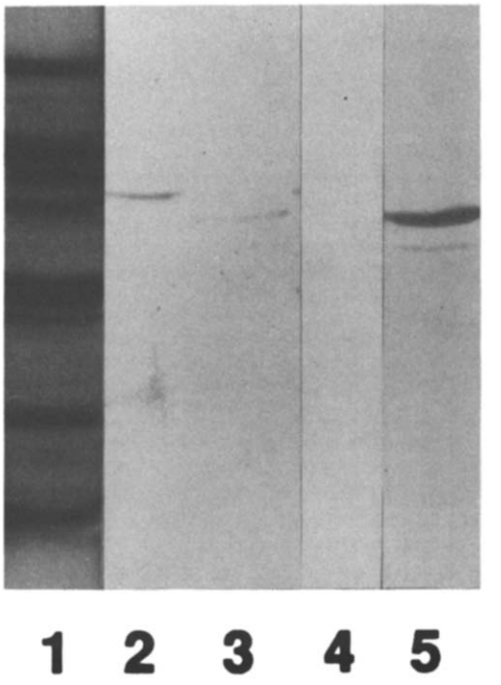

Fig. 5. Immunostaining of the pI 5.7 (putatively, aldose reductase) and pI 5.1 (putatively, aldehyde reductase) enzymes purified from human placenta. Purified enzymes (500 ng of each) were first subjected to SDS–PAGE. Following electrophoresis, they were electrotransferred to Immobilon-PDVF membranes. Immunodetection of the two enzymes was as described in Materials and Methods. Molecular weight standards (lane 1) were those listed in Fig. 3. Visualization of the molecular weight markers was with Coomassie Brilliant Blue R-250. Lanes 2 and 3: the pI 5.1 and 5.7 enzymes, respectively, probed with anti-human liver aldehyde reductase serum (1:1000 dilution). Lanes 4 and 5: the pI 5.1 and 5.7 enzymes, respectively, probed with anti-bovine lens aldose reductase serum (1:3000).

sulfate ions were included in the reaction mixture (data not shown).

Molecular weight values of 36,300 and 39,000 obtained under nondenaturing (data not presented) and denaturing (Fig. 3, lane 1) conditions, respectively, a pI value of 5.1 (Fig. 4, lane 3), and the observed substrate preference of the pI 5.1 enzyme (Table 2) are characteristic of human aldehyde reductase [13, 20–24]. Immunological recognition by anti-human liver aldehyde reductase serum (Fig. 5, lane 2) further confirms the identity of the pI 5.1 enzyme as aldehyde reductase.

The identification of the pI 5.7 enzyme as aldose reductase was based on the molecular weight values of 33,100 and 35,000 obtained under nondenaturing (data not presented) and denaturing (Fig. 3, lane 3) conditions, respectively, the pI value of 5.7, its substrate preference (Table 2), its activation by sulfate ions, and its immunological similarity to bovine lens aldose reductase (Fig. 5, lane 5), all of which are characteristic of human aldose reductase [12, 13, 20–25].

Aldose and aldehyde reductases each catalyzed the reduction of aldophosphamide. However, as judged by K_m values (0.15 vs 1.6 mM, Table 2), aldose reductase was more efficacious at doing so. In the placental sample used in this study, aldose and aldehyde reductases accounted for 94 and 6%, respectively, of the total aldo-keto reductase-catalyzed aldophosphamide (160 μM) reduction (Table 1).

As expected, known inhibitors of aldose and aldehyde reductases, viz. phenobarbital, sodium valproate, quercetin and sorbinil [26, 27], each inhibited the reduction of aldophosphamide catalyzed by these enzymes (Table 3). Sorbinil and, to a lesser extent, quercetin were the most potent in this regard. Inhibition of aldose reductase-catalyzed reduction of aldophosphamide by sorbinil was apparently noncompetitive (data not presented); the K_i value was 0.4 μM.

DISCUSSION

Although their specific biological/physiological

Table 2. Substrate preferences of the pI 5.7 (putatively, aldose reductase) and pI 5.1 (putatively, aldehyde reductase) reductases purified from human placenta*

Substrate (mM)†	pI 5.7 enzyme			pI 5.1 enzyme		
	K_m (mM)	V_{max} (μmol/min/mg protein)	V_{max}‡ (%)	K_m (mM)	V_{max} (μmol/min/mg protein)	V_{max}‡ (%)
DL-Glyceraldehyde (0.01–12.5)	0.04 ± 0.002	2.9 ± 0.04	100	4.8 ± 0.18	2.9 ± 0.05	100
D-Glucuronate (0.25–20)	6.7 ± 0.4	2.4 ± 0.09	83	3.1 ± 0.17	5.8 ± 0.1	200
D-Xylose (10–250)	15 ± 0.4	2.6 ± 0.02	90	981 ± 191	1.9 ± 0.3	66
D-Glucose (20–400)	98 ± 6.5	1.5 ± 0.06	52	§	§	§
Aldophosphamide (0.08–1.28)	0.15 ± 0.005	1.8 ± 0.02	62	1.6 ± 0.22	2.0 ± 0.2	69

* Kinetic constants were determined as described in Materials and Methods.

† Range of substrate concentrations utilized. Five to ten substrate concentrations were used to generate each pair of K_m and V_{max} (mean ± SEM) values.

‡ Percent of V_{max} value obtained when DL-glyceraldehyde was the substrate.

§ Not determined.

Table 3. Effects of various inhibitors on aldose reductase-catalyzed reduction of DL-glyceraldehyde and aldophosphamide and aldehyde reductase-catalyzed reduction of D-glucuronate and aldophosphamide

Inhibitor (μM)†	IC_{50}* (μM)			
	Aldose reductase		Aldehyde reductase	
	DL-Glyceraldehyde	Aldophosphamide	D-Glucuronate	Aldophosphamide
Phenobarbital (100–1000)	180	320	250	300
Sodium valproate (10–2000)	1550	1700	50	350
Quercetin (1–100)	2.4	3.6	18	26
Sorbinil (0.05–2)	0.23	0.46	>2‡	>2‡

* Inhibitor concentration that reduces enzyme activity to 50% of control. Control rates for aldose reductase-catalyzed reduction of DL-glyceraldehyde (10 mM) and aldophosphamide (0.16 mM) were 268 and 84 nmol/min, respectively. Control rates for aldehyde reductase-catalyzed reduction of D-glucuronate (10 mM) and aldophosphamide (0.16 mM) were 362 and 7.6 nmol/min, respectively.

† Range of inhibitor concentrations utilized. Five to twelve concentrations of each of the inhibitors were used to generate each IC_{50} value.

‡ Inhibition was observed but it was less than 50% at the highest concentration, viz. 2 μM, tested. Extrapolation of the values obtained indicate that 50% inhibition would have been achieved at about 3 μM.

raison d'etre is yet to be established, pyridine nucleotide-dependent aldo-keto reductases have been studied extensively, primarily because they may play important roles in the metabolism of several endogenous compounds. Thus, aldehyde reductase may be important in catalyzing the degradation of biogenic amines [28–30], aldose reductase may be important in retinoid metabolism [31] and/or in the conversion of glucose to sorbitol, the accumulation of which is associated with secondary diabetic complications such as nephropathy, neuropathy, retinopathy and cataractogenesis [26, 32], and carbonyl reductase may exert an *in vivo* regulatory function in prostaglandin metabolism [33–35].

Two of these enzymes, viz. aldose reductase and aldehyde reductase, are found in human placenta and were shown to catalyze the reduction of aldophosphamide to alcophosphamide. Aldose reductase was the more abundant of the two (*ca.* 5-fold) in the placental sample that we examined, and as judged by K_m values (0.15 vs 1.6 mM), was far more efficacious in catalyzing the reaction. Not surprisingly then, it was the more important of the

two in that it accounted for 94% of total pyridine nucleotide-dependent enzyme-catalyzed reduction of aldophosphamide (160 μM) to alcophosphamide effected by the placental sample that we examined.

Vander Jagt *et al.* [13] conducted experiments suggesting that the ratio of placental aldehyde reductase and aldose reductase ranges from 1:4 to 4:1, and that the total reductase activity in the different placenta samples varied considerably. The relative importance of aldehyde reductase in catalyzing the reduction of aldophosphamide to alcophosphamide would increase as the ratio of placental aldehyde reductase to aldose reductase content increased, but, given the K_m value of 1.6 mM characterizing the reaction, its absolute importance would never be great at pharmacological concentrations of aldophosphamide.

On the other hand, aldose reductase-catalyzed reduction of aldophosphamide to alcophosphamide may be of significance *in vivo* since the K_m value characterizing this reaction is 0.15 mM and aldose reductase is widely distributed in mammalian tissues [11–13, 20–25, 36–38]. Moreover, aldose reductase activity is elevated in a number of tissues, e.g. in erythrocytes, lens, brain, aorta, muscle and renal cells, during conditions of hyperglycemia and/or hyperosmolarity [38–44], and has been associated with severe, secondary diabetic complications [26, 32].

Multiple bands were observed when the placental aldose reductase was isoelectrofocused (Fig. 4). These bands were observed even when the purified enzyme was held in 5 mM dithiothreitol. Multiple banding could be due to the presence of (1) additional irrelevant proteins, or (2) several isoforms of aldose reductase. For several reasons, we believe the latter to be the case. First, Sephacryl S-200 gel filtration and SDS–PAGE each indicated homogeneity. Second, antibody to bovine lens aldose reductase recognized each of the bands (data not presented). Third, as judged by isoelectric focusing, at least two isoforms of aldose reductase have been found in lens tissues obtained from a number of mammalian species including humans [18, 45–47]. Activated and unactivated forms of a bovine aldose reductase exhibiting identical pI values, as judged by their behaviour on agarose isoelectric focusing, have also been reported [48]. Activated and unactivated forms of a human aldose reductase have also been reported [38]. Recent identification and characterization of a multigene family in rat, as well as in human, tissues coding for aldose reductase [49–51] suggest a basis for the polymorphism exhibited by human placental aldose reductase.

The metabolic fate of aldophosphamide includes enzyme-catalyzed oxidation to carboxyphosphamide in addition to enzyme-catalyzed reduction to alcophosphamide and β-elimination to phosphoramide mustard and acrolein (Fig. 1). In humans, oxidation of pharmacological concentrations of aldophosphamide to carboxyphosphamide is largely by two NAD(P)-dependent enzymes, viz. human aldehyde dehydrogenase-1 (ALDH-1) and succinic semialdehyde dehydrogenase [14]. The cytoplasmic concentration of NAD(P) is much greater than that of NAD(P)H [52]. Thus, all else being equal, the expectation is that enzyme-catalyzed oxidation of aldophosphamide will greatly exceed enzyme-catalyzed reduction of this metabolite. In accordance with this expectation, carboxyphosphamide is usually one of the major, and alcophosphamide is usually one of the minor, urinary metabolites in mammals given cyclophosphamide [reviewed in Refs. 1 and 2]. However, instead of the "normal" ALDH-1, variants that do not, or only poorly, catalyze the oxidation of acetaldehyde to acetic acid have been found in a few individuals [53–55]. In such people, enzyme-catalyzed reduction of aldophosphamide to alcophosphamide may be relatively more important if the ALDH-1 variant is also unable, or only poorly able, to catalyze the oxidation of aldophosphamide to carboxyphosphamide. Perhaps relevant in that regard is the report that less than 0.1% of the total dose was excreted as carboxyphosphamide in 4 of 14 patients injected with cyclophosphamide; excretion of alcophosphamide was not monitored in that investigation [56].

Hong and Chan [7] gave alcophosphamide to rats and found phosphoramide mustard in their plasma and urine. Formation of phosphoramide mustard from alcophosphamide could be direct or via aldophosphamide. Evidence in support of either scenario has yet to be generated. In any case, the observation of Hong and Chan [7] suggests that alcophosphamide, because it gives rise to phosphoramide mustard, will be toxic to tumor cells. Yet, at best, it is, in fact, only weakly cytotoxic, *vide supra*. These observations would be reconciled if conversion of alcophosphamide to phosphoramide mustard occurred largely outside of tumor and other target cells. According to this scenario, detoxification of aldophosphamide would be effected when it is reduced to alcophosphamide even though the reaction is actually or effectively reversible, i.e. whereas the reaction is actually or effectively reversible, detoxification is not. Thus, sensitivity to the oxazaphosphorines would decrease as cellular aldose reductase activity increased and vice versa. Not known is what the relative magnitude of aldose reductase expression is in critical normal, and neoplastic, cells.

Acknowledgements—This work was supported by USPHS Grant CA 21737 and Bristol-Myers Squibb Co. Grant 100-R220.

REFERENCES

1. Sladek NE, Metabolism of oxazaphosphorines. *Pharmacol Ther* **37**: 301–355, 1988.
2. Sladek NE, Metabolism and pharmacokinetic behavior of cyclophosphamide and related oxazaphosphorines. In: *Metabolism and Reactions of Anticancer Drugs* (*The International Encyclopedia of Pharmacology and Therapeutics*) (Ed. Powis G), in press.
3. Bakke JE, Feil VJ, Fjelstul CE and Thacker EJ, Metabolism of cyclophosphamide by sheep. *J Agric Food Chem* **20**: 384–388, 1972.
4. Connors TA, Cox PJ, Farmer PB, Foster AB and Jarman M, Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. *Biochem Pharmacol* **23**: 115–129, 1974.

5. Struck RF, Kirk MC, Witt MH and Laster WR Jr, Isolation and mass spectral identification of blood metabolites of cyclophosphamide: Evidence for phosphoramide mustard as the biologically active metabolite. *Biomed Mass Spectrom* **2**: 46–52, 1975.
6. Domeyer BE and Sladek NE, Kinetics of cyclophosphamide biotransformation *in vivo*. *Cancer Res* **40**: 174–180, 1980.
7. Hong PS and Chan KK, Identification and quantitation of alcophosphamide, a metabolite of cyclophosphamide, in the rat using chemical ionization mass spectrometry. *Biomed Environ Mass Spectrom* **14**: 167–172, 1987.
8. Hong PS, Srigritsanapol A and Chan KK, Pharmacokinetics of 4-hydroxycyclophosphamide and metabolites in the rat. *Drug Metab Dispos* **19**: 1–7, 1991.
9. Friedman OM, Wodinsky I and Myles A, Cyclophosphamide (NSC-26271)-related phosphoramide mustards—Recent advances and historical perspective. *Cancer Treat Rep* **60**: 337–346, 1976.
10. Montgomery JA and Struck RF, Synthesis and structure–activity relationships of pre-activated analogs of cyclophosphamide (NSC-26271). *Cancer Treat Rep* **60**: 381–393, 1976.
11. Wermuth B and von Wartburg J-P, Aldose reductase from human tissue. In: *Methods in Enzymology* (Ed. Wood WA), Vol. 89, pp. 181–186. Academic Press, New York, 1982.
12. Vander Jagt DL, Stangebye LA, Hunsaker LA, Eaton RP and Sibbitt WL Jr, Purification of aldose reductase from human placenta and stabilization of the inhibitor binding site. *Biochem Pharmacol* **37**: 1051–1056, 1988.
13. Vander Jagt DL, Hunsaker LA, Robinson B, Stangebye LA and Deck LM, Aldehyde and aldose reductases from human placenta. Heterogeneous expression of multiple enzyme forms. *J Biol Chem* **265**: 10912–10918, 1990.
14. Dockham PA, Lee M-O and Sladek NE, Identification of human liver aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde. *Biochem Pharmacol* **43**: 2453–2469, 1992.
15. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
16. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
17. Sambrook J, Fritsch EF and Maniatis T, Transfer of proteins from SDS–polyacrylamide gels to solid supports: Immunological detection of immobilized proteins (western blotting). *Molecular Cloning: A Laboratory Manual*, 2nd Edn, pp. 18.60–18.75. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
18. Petrash JM, DeLucas LJ, Bowling E and Egen N, Resolving isoforms of aldose reductase by preparative isoelectric focusing in the Rotofor. *Electrophoresis* **12**: 84–90, 1991.
19. Wilkinson GN, Statistical estimations in enzyme kinetics. *Biochem J* **80**: 324–332, 1961.
20. Das B and Srivastava SK, Purification and properties of aldose reductase and aldehyde reductase II from human erythrocyte. *Arch Biochem Biophys* **238**: 670–679, 1985.
21. Das B, Song HP, Ansari NH, Hair GA and Srivastava SK, Purification and properties of aldose reductase and aldehyde reductase II from human lens. *Lens Res* **4**: 309–335, 1987.
22. Nakayama T, Tanimoto T and Kador PF, Human erythrocyte aldose and aldehyde reductase. In: *Progress in Clinical and Biological Research* (Eds. Weiner H and Flynn TG), Vol. 290, pp. 265–277. Alan R. Liss, New York, 1989.
23. Ansari NH, Bhatnagar A, Liu S and Srivastava SK, Purification and characterization of aldose reductase and aldehyde reductase from human kidney. *Biochem Int* **25**: 755–765, 1991.
24. Tanimoto T, Ohta M, Tanaka A, Ikemoto I and Machida T, Purification and characterization of human testis aldose and aldehyde reductase. *Int J Biochem* **23**: 421–428, 1991.
25. Morjana NA and Flynn TG, Aldose reductase from human psoas muscle. Purification, substrate specificity, immunological characterization, and effects of drugs and inhibitors. *J Biol Chem* **264**: 2906–2911, 1989.
26. Kador PF, Pharmacology of aldose reductase inhibitors. *Med Res Rev* **8**: 325–352, 1988.
27. Sato S and Kador PF, Inhibition of aldehyde reductase by aldose reductase inhibitors. *Biochem Pharmacol* **40**: 1033–1042, 1990.
28. Wermuth B and Munch JDB, Reduction of biogenic aldehydes by aldehyde reductase and alcohol dehydrogenase from human liver. *Biochem Pharmacol* **28**: 1431–1433, 1979.
29. Whittle SR and Turner AJ, Biogenic aldehyde metabolism in rat brain. Differential sensitivity of aldehyde reductase isoenzymes to sodium valproate. *Biochim Biophys Acta* **657**: 94–105, 1981.
30. Helander A and Tottmar O, Metabolism of biogenic aldehydes in isolated human blood cells, platelets and in plasma. *Biochem Pharmacol* **36**: 1072–1082, 1987.
31. Sladek NE and Lee M-O, The use of immortalized mouse L1210/OAP cells established in culture to study the major class 1 aldehyde dehydrogenase-catalyzed oxidation of aldehydes in intact cells. In: *Enzymology and Molecular Biology of Carbonyl Metabolism 4* (Eds. Weiner H, Crabb DW and Flynn TG), pp. 51–62. Plenum Press, New York, 1993.
32. Kador PF, Terubayashi H, Millen J and Kinoshita JH, The role of aldose reductase in the development of diabetic complications. In: *Polyol Pathway and Its Role in Diabetic Complications* (Eds. Sakamoto N, Kinoshita JH, Kador PF and Holta N), pp. 20–31. Elsevier, Amsterdam, 1988.
33. Wermuth B, Purification and properties of an NADPH-dependent carbonyl reductase from human brain. Relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase. *J Biol Chem* **256**: 1206–1213, 1981.
34. Inazu N, Ruepp B, Wirth H and Wermuth B, Carbonyl reductase from human testis: Purification and comparison with carbonyl reductase from human brain and rat testis. *Biochim Biophys Acta* **1116**: 50–56, 1992.
35. Schieber A, Frank RW and Ghisla S, Purification and properties of prostaglandin 9-ketoreductase from pig and human kidney. Identity with human carbonyl reductase. *Eur J Biochem* **206**: 491–502, 1992.
36. Conrad SM and Doughty CC, Comparative studies on aldose reductase from bovine, rat and human lens. *Biochim Biophys Acta* **708**: 348–357, 1982.
37. Srivastava SK, Ansari NH, Hair GA and Das B, Aldose and aldehyde reductases in human tissues. *Biochim Biophys Acta* **800**: 220–227, 1984.
38. Srivastava SK, Hair GA and Das B, Activated and unactivated forms of human erythrocyte aldose reductase. *Proc Natl Acad Sci USA* **82**: 7222–7226, 1985.
39. Srivastava SK, Ansari NH, Hair GA, Jaspan J, Rao MB and Das B, Hyperglycemia-induced activation of human erythrocyte aldose reductase and alterations in kinetic properties. *Biochim Biophys Acta* **870**: 302–311, 1986.
40. Bagnasco SM, Uchida S, Balaban RS, Kador PF and

Burg MB, Induction of aldose reductase and sorbitol in renal inner medullary cells by elevated extracellular NaCl. *Proc Natl Acad Sci USA* **84**: 1718–1720, 1987.
41. Garcia-Perez A, Martin B, Murphy HR, Uchida S, Murer H, Cowley BD Jr, Handler JS and Burg MB, Molecular cloning of cDNA coding for kidney aldose reductase. Regulation of specific mRNA accumulation by NaCl-mediated osmotic stress. *J Biol Chem* **264**: 16815–16821, 1989.
42. Moriyama T, Garcia-Perez A and Burg MB, Osmotic regulation of aldose reductase protein synthesis in renal medullary cells. *J Biol Chem* **264**: 16810–16814, 1989.
43. Petrash JM, Flath M, Sens D and Bylander J, Effects of osmotic stress and hyperglycemia on aldose reductase gene expression in human renal proximal tubule cells. *Biochem Biophys Res Commun* **187**: 201–208, 1992.
44. Tawata M, Ohtaka M, Hosaka Y and Onaya T, Aldose reductase mRNA expression and its activity are induced by glucose in fetal rat aortic smooth muscle (A10) cells. *Life Sci* **51**: 719–726, 1992.
45. Markus HB, Raducha M and Harris H, Tissue distribution of mammalian aldose reductase and related enzymes. *Biochem Med* **29**: 31–45, 1983.
46. Tanimoto T, Fukuda H and Kawamura J, Purification and some properties of aldose reductase from rabbit lens. *Chem Pharm Bull (Tokyo)* **31**: 2395–2403, 1983.
47. Sato S and Kador PF, Rat lens aldehyde reductase. *Invest Ophthalmol Vis Sci* **30**: 1618–1622, 1989.
48. Grimshaw CE, Shahbaz M, Jahangiri G, Putney CG, McKercher SR and Mathur EJ, Kinetic and structural effects of activation of bovine kidney aldose reductase. *Biochemistry* **28**: 5343–5353, 1989.
49. Bohren KM, Bullock B, Wermuth B and Gabbay KH, The aldo-keto reductase superfamily. cDNAs and deduced amino acid sequences of human aldehyde and aldose reductases. *J Biol Chem* **264**: 9547–9551, 1989.
50. Nishimura C, Wistow G and Carper D, Rat lens aldose reductase: Characterization of its primary structure, mRNA, and gene complexity. In: *Progress in Clinical and Biological Research* (Eds. Weiner H and Flynn TG), Vol. 290, pp. 211–220. Alan R. Liss, New York, 1989.
51. Graham C, Szpirer C, Levan G and Carper D, Characterization of the aldose reductase-encoding gene family in rat. *Gene* **107**: 259–267, 1991.
52. Krebs HA and Veech RL, Equilibrium relations between pyridine nucleotides and adenine nucleotides and their roles in the regulation of metabolic processes. In: *Advances In Enzyme Regulation* (Ed. Weber G), Vol. 7, pp. 397–413. Pergamon Press, Oxford, 1969.
53. Yoshida A, Wang G and Davé V, Determination of genotypes of human aldehyde dehydrogenase $ALDH_2$ locus. *Am J Hum Genet* **35**: 1107–1116, 1983.
54. Eckey R, Agarwal DP, Saha N and Goedde HW, Detection and partial characterization of a variant form of cytosolic aldehyde dehydrogenase isozyme. *Hum Genet* **72**: 95–97, 1986.
55. Yoshida A, Davé V, Ward RJ and Peters TJ, Cytosolic aldehyde dehydrogenase (ALDH1) variants found in alcohol flushers. *Ann Hum Genet* **53**: 1–7, 1989.
56. Hadidi A-HFA, Coulter CEA and Idle JR, Phenotypically deficient urinary elimination of carboxyphosphamide after cyclophosphamide administration to cancer patients. *Cancer Res* **48**: 5167–5171, 1988.