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Bovine lens aldehyde reductase (aldose reductase)

Purification, kinetics and mechanism

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Aldehyde reductase (aldose reductase) was purified to homogeneity (as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) from bovine lens by affinity chromatography on NADP$^+$–Sepharose. The enzyme, a monomer of M_r about 40000, was active with a variety of α-hydroxyketones, including fructose. The minimum degree of the rate equation was 2:2 in the case of DL-glyceraldehyde, but linear kinetics were observed for glucose and NADPH over the concentration range studied. The enzyme largely followed a ternary-complex mechanism, with initial binding of NADPH before glucose and final release of NADP$^+$.

Bovine lens aldose reductase is one of two classes of aldehyde reductases in mammalian cells (see Crabbe *et al.*, 1982; Flynn, 1982; Turner, 1982).

The enzyme has been purified to homogeneity (Sheaff & Doughty, 1976; Conrad & Doughty, 1982), and it has been suggested that the enzyme obeys a random mechanism with DL-glyceraldehyde as substrate at pH 7.0 (Doughty & Conrad, 1982).

Considerable interest in the enzyme has been generated because of its potential role in the development of cataract in diabetics (van Heyningen, 1959; Varma & Kinoshita, 1974; Crabbe *et al.*, 1982; Kador *et al.*, 1982; Datiles *et al.*, 1982). In the present paper we report the purification of the enzyme by affinity chromatography, its activity with a variety of α-hydroxyketones, its inhibition by putative aldose reductase inhibitors, and its kinetic mechanism with D-glucose and NADPH.

Materials and methods

Materials

The monobarium salt of diethylacetal glyceraldehyde 3-phosphate, diethyl pyrocarbonate, *N*-ethylmaleimide, Li_2SO_4, Blue Dextran, D-sorbitol, phenazine methosulphate, Nitro Blue Tetrazolium, succinic semialdehyde and *p*-nitrobenzaldehyde were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

β-NADPH (type X) and β-NADP$^+$ (Sigma grade) were from Sigma Chemical Co. β-NAD$^+$ (grade 1) and β-NADH (grade 1) were purchased from Boehringer Corp (Lewes, East Sussex, U.K.). Glyceraldehyde 3-phosphate dehydrogenase and yeast alcohol dehydrogenase were also from Boehringer Corp.

DL-Glyceraldehyde, phenylglyoxal, tetranitromethane and bis-dimethylacetal ester of malondialdehyde were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Malondialdehyde was prepared from its ester by the method of Kikugawa *et al.* (1980).

D-Glucose, *p*-chloromercuribenzoate and protein markers for SDS/polyacrylamide-gel electrophoresis were obtained from BDH Chemicals (Poole, Dorset, U.K.).

All other chemicals used were obtained from BDH Chemicals and were of the highest purity obtainable.

Determination of protein

Protein was determined either by the method of Lowry *et al.* (1951) or by the method of Bradford (1976). Bovine serum albumin was used as a standard.

Assay of enzyme activities

All enzyme assays were carried out at 37°C (unless otherwise stated) on a Perkin–Elmer 550 UV–Vis double-beam recording spectrophotometer fitted with an automatic cell-changer; 1 ml or 3 ml non-reflecting quartz or glass cuvettes were used

Abbreviation used: SDS, sodium dodecyl sulphate.

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throughout. The Finpipettes used had an accuracy of ±3%.

Aldehyde reductase activity was assayed in 1 ml of 100mM-potassium phosphate buffer, pH6.2, containing NADPH (50 μM) and either DL-glyceraldehyde (5mM) or glucose (80mM) unless stated otherwise. The assay was started by addition of enzyme.

Polyol dehydrogenase activity was assayed in 1 ml of 100mM-Tris/HCl buffer, pH8.9, containing sorbitol (40mM) and NAD^+ (1.5mM). The assay was started by adding the enzyme to both blank and test cuvettes. The blank contained all reagents except sorbitol.

Where the effect of pH on enzyme activity was studied, buffers of different pH values but of the same ionic strength were used. The enzyme rates for both aldehyde reductase and polyol dehydrogenase were linear for at least 8min. One unit of enzyme is defined as that amount catalysing the oxidation/reduction of 1 μmol of substrate/min at 37°C.

NADPH oxidation is accelerated in phosphate buffer at around pH6.8 (Lowry *et al.*, 1961). This breakdown was compensated for by including NADPH in both blank and test. The addition of DL-glyceraldehyde to NADPH in phosphate buffer did not stimulate the breakdown of NADPH for about 15min. The addition of lens homogenate to the assay mixture containing NADPH and buffer was found to increase the NADPH oxidation by 10%, and this was corrected when homogenate was used by using an enzyme blank where the enzyme was added to both blank and test. An oxidation of 1.2%/h for NADPH (measured in terms of ΔA_{340}) was observed in phosphate buffer. NADPH solution was always made fresh and used immediately.

Computation

The rates shown in all Figures and Tables are the means ± S.D. for two to four determinations. Where no bar units are shown on Figures the standard deviations are within the plotted points. A linear-regression analysis was performed to find the slope and intercept values from the best-fit straight lines for all linear sets of initial-velocity data. Kinetic parameters were calculated by using a BMDP3R non-linear regression program on an ICL 2980 computer, or by the computer methods of Crabbe (1982, 1984).

Enzyme purification

All steps were carried out at 4°C, and fractions were assayed for NADPH oxidation by both DL-glyceraldehyde and D-glucose.

Bovine eyes were collected from the slaughterhouse (Alf Meade and Sons, Reading, Berks., U.K.) on the day the animals were slaughtered. Lenses were extracted the same day and stored at −30°C. Lenses (average weight 1.9 ± 0.2g) were mechanically homogenized at 4°C in 5vol. of 50mM-potassium phosphate buffer, pH7.2, containing 2-mercaptoethanol (5mM). The homogenate was then centrifuged at 10000*g* for 20min at 4°C in an MSE centrifuge. The pellet, containing cellular debris, was discarded.

The lens homogenate after centrifugation at 10000*g* was adjusted to 60% saturation by adding solid $(NH_4)_2SO_4$, slowly with stirring. After 1h the solution was centrifuged at 10000*g* for 20min. The supernatant was adjusted to 85% saturation with $(NH_4)_2SO_4$ and treated as before. The pellet was dissolved in a minimum volume of 50mM-potassium phosphate buffer, pH7.2, containing 2-mercaptoethanol (5mM) and dialysed overnight against 2 litres of the same buffer, with one change. After dialysis, enzyme was concentrated against solid sucrose. Sucrose and $(NH_4)_2SO_4$ were further removed by chromatography on a column (24cm × 3cm) of Sephadex G-25.

The enzyme fraction was exhaustively dialysed against 100mM-potassium phosphate buffer, pH6.2, containing 2-mercaptoethanol (10mM) and NaN_3 (0.02%), and 50mg was applied to a column (15cm × 0.6cm) of $NADP^+$–Sepharose prepared as described previously (Axen *et al.*, 1967; Mosbach *et al.*, 1972). After 30min the column was washed with 100mM-potassium phosphate buffer, pH6.2, containing 0.4M-NaCl until the A_{280} of the buffer wash was zero. Enzyme was then eluted with NADPH (0.4mM). The flow rate of the column was 8ml/h. Fractions containing enzyme activity were pooled and dialysed to remove NADPH.

SDS/polyacrylamide-gel electrophoresis

This was performed as described by Laemmli & Favre (1973).

Amino acid analysis

This was performed as described previously (Ting & Crabbe, 1983).

Results and discussion

Polyol-pathway activities in lens of various species

Much interest has been raised concerning lens aldose reductase because of its supposed role in the development of diabetic cataracts. This role has been ascribed on the basis of experiments carried out in animals, mainly rodents. Assay of aldose (aldehyde) reductase and polyol dehydrogenase at their pH optima shows that there is considerable variation in enzyme activities between species. With D-glucose as substrate, specific activities (munits/mg of protein) for aldehyde reductase

were as follows: human, 0.12; ox, 0.04; rabbit, 0.18; rat, 0.8; mouse, 0.01. Polyol dehydrogenase activity could not be detected in rabbit, rat or mouse lenses, but specific activities in human and ox lenses were 4.0 and 0.12units/mg respectively. Mean values for four lenses are quoted, with variations less than 10%. When DL-glyceraldehyde was used as substrate, aldehyde reductase specific activities were generally 3-fold higher. Ages of the animals were: human, 50–60 years; ox, 2–4 years; rat, 5 weeks; rabbit, 5 months; mouse, 5 weeks. These findings indicate that testing anti-cataract drugs with animal models may not give an accurate indication of polyol-pathway activity. Additional complicating factors are that sorbitol can easily be formed from fructose via the reverse reaction catalysed by polyol dehydrogenase in the human (Jedziniak *et al.*, 1981), that non-enzymic reactions including glycosylation become important in diabetes (see Harding & Crabbe, 1984), and that monosaccharides themselves can autoxidize under physiological conditions to produce reactive dicarbonyl compounds and free radicals (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*a*,*b*).

Enzyme purification

Table 1 shows the results of the enzyme purification. The enzyme appeared homogeneous by SDS/polyacrylamide-gel electrophoresis. Similar homogeneous preparations of activity were obtained by gel filtration, ion-exchange chromatography and affinity chromatography on Procion Red–agarose. When D-glucose was used as substrate, activity was co-eluted with DL-glyceraldehyde-reducing activity. We were not able to separate the activities with D-glucose or DL-glyceraldehyde as substrates.

All kinetic experiments were performed with enzyme that appeared homogeneous by SDS/polyacryamide-gel electrophoresis.

Enzyme characterization

(*a*) M_r. The pure enzyme had M_r 40000 ± 2000 judged by SDS/polyacrylamide-gel electrophoresis. Gel filtration on a calibrated Sephadex G-200 column (120cm × 0.9cm) gave a lower M_r value (32000). No significant variation with method of preparation was noted. A similar discrepancy has been reported by Sheaff & Doughty (1976) for their preparation of bovine lens aldehyde reductase. Our value of 40000 is similar to that obtained by Cromlish & Flynn (1983) for pig kidney aldehyde reductase.

No significant minor forms of lens enzyme activity were detected. Indeed, EDTA was included in appropriate buffers, as suggested by Cromlish & Flynn (1983) to largely prevent any proteolytic-cleavage product in their purification of pig kidney aldehyde reductase.

(*b*) *Amino acid composition.* The amino acid composition of the enzyme was similar to those reported for other aldehyde reductases (Crabbe *et al.*, 1982). The cysteine content (corrected) determined by performic acid oxidation was found to be 8 residues/1000 residues.

(*c*) *Effect of pH.* Fig. 1 shows the relationships between enzyme activity and pH with DL-glyceraldehyde and D-glucose as substrates. All the buffers, citrate, acetate, succinate and cacodylate, that were tried below 5.9 were found to be strong inhibitors of the enzyme with both glyceraldehyde and glucose as aldehyde substrates.

(*d*) *Effect of ionic strength.* With both $(NH_4)_2SO_4$ and Li_2SO_4 the enzyme activity increased 1.8-fold at around I 0.6, the activating effect declined above I 0.6 and then the activity slowly resumed its original value at I 0.85. With NH_4Cl there was little (with glucose as substrate) or no (with glyceraldehyde as substrate) activating effect on the enzyme. At above I 0.2, NH_4Cl exerted an inhibitory effect on the enzyme. With KCl, there was a slight activating effect on the glyceraldehyde-reducing activity up to about I 0.4, but the activity then gradually reverted to its original value with further increase in I. With both DL-glyceraldehyde and D-glucose, the effects of ions were very similar.

(*e*) *Effect of temperature.* The activation energies calculated from Arrhenius plots were 58.8kJ/mol for DL-glyceraldehyde as substrate and 64.7kJ/mol for D-glucose as substrate.

Steady-state kinetics

Eadie–Hofstee plots for D-glucose and NADPH were linear over 100-fold concentration ranges.

Table 1. *Purification of aldehyde reductase involving the use of chromatography on NADP$^+$–Sepharose*

DL-Glyceraldehdye was used as substrate. For details see the text.

	Total protein (mg)	Total activity (units)	Specific activity (munits/mg of protein)	Purification factor	Yield (%)
Crude extract	7140	1.1	0.15	1	100
60–85%-satn. $(NH_4)_2SO_4$	300	0.5	1.66	11	45
NADP$^+$–Sepharose	1.2	0.24	200	1333	22

Substrate inhibition was observed with *p*-nitrobenzaldehyde (above 2mM), DL-glyceraldehyde (above 20mM). DL-glyceraldehyde 3-phosphate (above 1mM) and malondialdehyde (above 0.8mM). Maximum non-linearity was shown by DL-glyceraldehyde (Fig. 2), where computer curve-fitting indicated that the minimum degree of the rate equation was 2:2. Table 2 gives the kinetic parameters of the enzyme with a number of substrates. The enzyme acts on keto groups (fructose) as well as on aldehyde groups (glucose). Vitamin K_3 (menadione) could also act as a substrate.

Enzyme mechanism

D-Glucose and NADPH were used as substrates. Double-reciprocal plots and slope and intercept replots were linear in the absence of products (Figs. 3*a* and 3*b*). $NADP^+$ showed competitive inhibition against NADPH and non-competitive inhibition against D-glucose (Figs. 4*a* and 4*b*). Sorbitol showed non-competitive inhibition with both glucose and $NADP^+$. Similar inhibition patterns were obtained at saturating concentrations of glucose.

The initial-velocity studies are consistent with an ordered ternary-complex mechanism with initial binding of NADPH, which can be described by the following equation:

$$v = \frac{V_{max.}[A][B]}{K_s^A K_m^B + K_m^A[B] + K_m^B[A] + [A][B]}$$

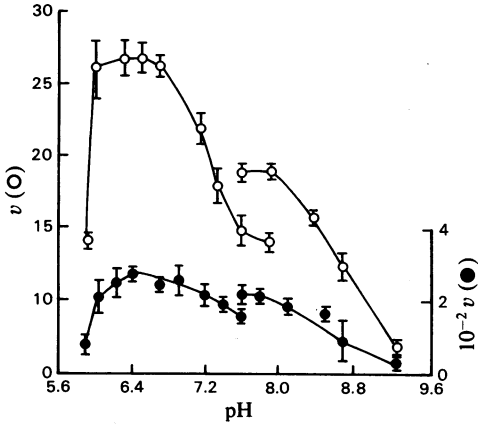

Fig. 1. *Effect of pH on highly purified bovine lens aldehyde reductase activity with* DL-*glyceraldehyde* (○) *and* D-*glucose* (●) *as substrates*
For details see the text. *v* is expressed as μmol/min.

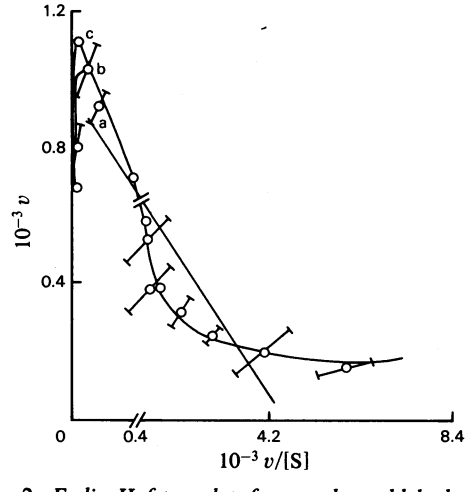

Fig. 2. *Eadie–Hofstee plot for* DL-*glyceraldehyde with highly purified bovine lens aldehyde reductase*
○, Data from observations. Curves a, b and c represent the best-fit computer-predicted curves for 1:1, 2:2 and 3:3 functions respectively. For details see the text.

Table 2. *Kinetic parameters for bovine lens aldehyde reductase*
N.D., Not detectable.

Substrate	K_m (mM)	$V_{max.}$ (μmol/min per mg of enzyme)	$k_{cat.}$ (s^{-1})	$10^3 \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
p-Nitrobenzaldehyde	0.03	0.54	0.27	9
DL-Glyceraldehyde	0.123	1.27	0.77	6.41
D-Glucose	120	0.5	0.3	0.0025
D-Glucuronate	4.3	0.42	0.25	0.058
3-*O*-Methylglucose	150	0.23	0.06	0.0004
Fructose	23	0.02	0.015	0.0065
Glyceraldehyde 3-phosphate	0.2	0.014	0.08	0.4
Malondialdehyde	0.25	0.19	0.13	0.52
Succinic semialdehyde	N.D.	N.D.		
L-Glucose	N.D.	N.D.		
NADPH	0.009	0.49	0.28	31.0
NADH	1.64	0.0003		

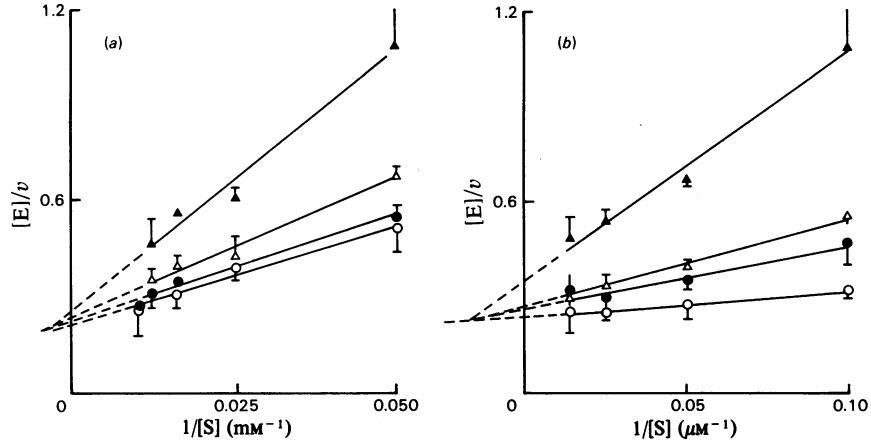

Fig. 3. *Effect of varying the concentration of both substrates of bovine lens aldehyde reductase on initial rates*
(*a*) D-Glucose as the varied substrate (S) at fixed NADPH concentrations: ○, 10 μM; ●, 20 μM; △, 40 μM; ▲, 80 μM. (*b*) NADPH as the varied substrate at fixed D-glucose concentrations: ○, 20 mM; ●, 40 mM; △, 80 mM; ▲, 100 mM. *v* is the initial velocity in μmol/min and [E] is the amount of enzyme in μmol.

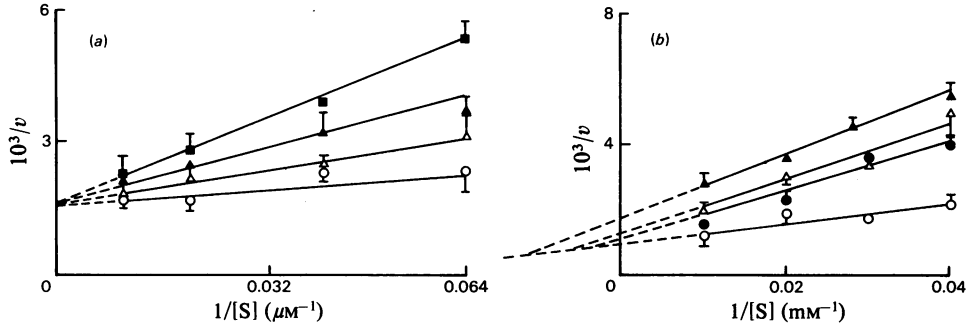

Fig. 4. *Inhibition of initial velocities of bovine lens aldehyde reductase in the presence of products*
(*a*) NADPH as the varied substrate (S), with 80 mM-D-glucose, at fixed concentrations of $NADP^+$: ○, no product present; △, 30 μM; ▲, 50 μM; ■, 70 μM. (*b*) D-Glucose as the varied substrate, with 50 μM-NADPH, at fixed concentrations of $NADP^+$: ○, no product present; ●, 50 μM; △, 70 μM; ▲, 90 μM.

The kinetic parameters were calculated to be $V_{max.} = 0.01\,\mu mol/min$ per mg, $K_s^{NADPH} = 5.41 \pm 0.5\,\mu M$; $K_m^{NADPH} = 8.2 \pm 1.0\,\mu M$ and $K_m^{glucose} = 21.8 \pm 3\,mM$. The K_{ii} ($K_{i(intercept)}$) and K_{is} ($K_{i(slope)}$) for product inhibitors are given in Table 3. With NADPH at low concentrations, $NADP^+$ showed a hyperbolic slope effect, but, at a near-saturating concentration of NADPH, $NADP^+$ showed a hyperbolic intercept effect with glucose as variable substrate. The hyperbolic slope effect was prominent at high concentration (70 μM) of $NADP^+$, but as the $NADP^+$ concentration was lowered to 50 μM the K_{is} decreased to 5 μM, the dissociation constant for NADPH. With glucose as the variable substrate, a similar hyperbolic intercept effect at high $NADP^+$ concentrations (90 μM) was observed, but at lower $NADP^+$ concentration (70 μM) the K_{ii} decreased to 5 μM. This indicates that a minor random pathway may be operating at high $NADP^+$ concentrations. However, the enzyme does not show the same random behaviour with D-glucose as has been reported with DL-glyceraldehyde (Doughty & Conrad, 1982). One factor that may be influential in the apparent random behaviour with DL-glyceraldehyde is the autoxidation of that compound under the assay conditions used. We have shown that DL-glyceraldehyde can autoxidize under physiological conditions to give rise to free radicals and dicarbonyl compounds (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*a*,*b*), and that these may influence the oxidation of the NADPH that is bound to the protein.

Table 3. *Kinetic parameters for product inhibitors of purified bovine lens aldehyde reductase*

K_{ii} and K_{is} were determined from the replots of intercepts and slopes against inhibitor concentration.

Inhibitor	Variable substrate	Fixed substrate	Inhibition	K_{ii}	K_{is}
Sorbitol	Glucose	NADPH	Non-competitive	1.9M	1.7M
Sorbitol	NADPH	Glucose	Non-competitive	2.25M	2.8M
$NADP^+$	Glucose	NADPH	Non-competitive	135μM*	30μM
$NADP^+$	NADPH	Glucose	Competitive	—	60μM*

* Values determined from the tangents to the hyperbolic curves.

Table 4. *Inhibition constants for Sulindac, compound FPL 58665, Procion Red, nicotinamide and adenosine 2',5'-bisphosphate*

Inhibitor	Variable substrate	K_{ii} (μM)	K_{is} (μM)
Sulindac	DL-Glyceraldehyde	0.38	0.5
	D-Glucose	0.9	1.05
	NADPH	1.5	*
Compound FPL 58665	DL-Glyceraldehyde	2	8
	D-Glucose	1.5	3.2
	NADPH	6.6	*
Procion Red	NADPH	†	3.5
Adenosine 2',5'-bisphosphate	NADPH	†	20
Nicotinamide	NADPH	†	10

* Non-linear slope effect.
† No intercept effect.

The rate of autoxidation of DL-glyceraldehyde is considerably greater than that of D-glucose, and the products of the autoxidation may be responsible for the non-linear kinetics observed with DL-glyceraldehyde with this enzyme (Fig. 2) and with a similar protein from the human erythrocyte (Crabbe *et al.*, 1981).

Effects of inhibitors

Procion Red, nicotinamide and adenosine 2',5'-bisphosphate all acted as competitive inhibitors with NADPH as the variable substrate. Sodium barbitone (5mM) did not inhibit the enzyme with either DL-glyceraldehyde or D-glucose as substrate. Sulindac (2-{5-fluoro-2-methyl-1-[*p*-(methylsulphinyl)benzylidine}indene-3-acetic acid), an anti-inflammatory drug, and compound FPL 58665 {disodium 4,6-dioxo-10-propyl-4*H*,6*H*-pyrano[3,2-*g*]-quinoline-2,8-dicarboxylate}, an analogue of disodium cromoglycate, acted as non-competitive inhibitors against DL-glyceraldehyde, D-glucose and NADPH. All intercept replots were linear, but non-linear slope replots with NADPH indicated either that the inhibitors could bind to more than one site on the enzyme, or that at high concentration inhibitors reacted with NADPH. Table 4 shows the inhibition constants for the reversible inhibitors studied.

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