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# Kinetic mechanism of sheep liver NADPH-dependent aldehyde reductase

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The kinetic mechanism of the major sheep liver aldehyde reductase (ALR1) was studied with three aldehyde substrates: *p*-nitrobenzaldehyde, pyridine-3-aldehyde and D-glucuronate. In each case the enzyme mechanism was sequential and product-inhibition studies were consistent with an ordered Bi Bi mechanism, with the coenzymes binding to the free enzyme. Binding studies were used to investigate the interactions of substrates, products and inhibitors with the free enzyme. These provided evidence for the binding of D-glucuronate, L-gulonate and valproate, as well as $NADP^+$ and NADPH. The enzyme was inhibited by high concentrations of D-glucuronate in a non-competitive manner, indicating that this substrate was able to bind to the free enzyme and to the $E \cdot NADP^+$ complex at elevated concentrations. Although the enzyme was inhibited by high pyridine-3-aldehyde concentrations, there was no evidence for the binding of this substrate to the free enzyme. Sheep liver ALR1 was inhibited by the ionized forms of alrestatin, sorbinil, valproate, 2-ethylhexanoate and phenobarbitone, indicating the presence of an anion-binding site similar to that described for the pig liver enzyme, which interacts with inhibitors and substrates containing a carboxy group. Sorbinil, valproate and 2-ethylhexanoate inhibited the enzyme uncompetitively at low concentrations and non-competitively at high concentrations, whereas phenobarbitone and alrestatin were non-competitive and uncompetitive inhibitors respectively. The significance of these results with respect to inhibitor and substrate binding is discussed.

## INTRODUCTION

The aldehyde reductases comprise a group of enzymes that catalyse the conversion of a wide range of aldehydes and ketones into their corresponding alcohols. These enzymes have been isolated from a wide variety of tissues, including kidney, brain and liver (for a review see Flynn, 1982). There are two major forms of NADPH-specific aldehyde reductase present in mammalian tissues, designated ALR1 and ALR2, and having high and low $K_m$ values respectively for the substrate *p*-nitrobenzaldehyde (Turner & Flynn, 1982). ALR1 appears to be synonymous with hexonate dehydrogenase and daunorubicin reductase. Several investigations have been carried out in order to elucidate the kinetic mechanism of ALR1 from a number of sources. However, these studies have yielded conflicting results. Earlier work indicated that the rat skeletal-muscle enzyme followed a ping-pong mechanism (Toews, 1967), whereas a random mechanism was obeyed by the bovine brain enzyme (Bronaugh & Erwin, 1972). More-recent studies have indicated a compulsory-order mechanism with NADPH binding first, although differences in the order of product release have been reported. Product release from the human liver (Wermuth & Von Wartburg, 1980) and pig kidney (Morpeth & Dickinson, 1981) enzymes has been described as random. In contrast, Prairie & Lai (1973) and Davidson & Flynn (1979) interpreted their results as indicating an ordered product release from this latter enzyme, and product release from the rat brain (Rivett & Tipton, 1981) and ox kidney (Daly & Mantle, 1982) enzymes is also ordered.

Tipton *et al.* (1982) suggested that these differences may reflect intrinsic differences in the source of the enzyme studied, differences in the assay conditions employed by various workers, or the use of different substrates to assay the enzyme. Indeed, the results reported by Branlant *et al.* (1981) and Magnien & Branlant (1983) suggested that the kinetic mechanism of pig liver aldehyde reductase varied according to the nature of the substrate.

Since there was some uncertainty concerning the kinetic mechanism of ALR1, the mechanism of the sheep liver enzyme was studied in detail with an aromatic aldehyde substrate (*p*-nitrobenzaldehyde), with an aliphatic aldehyde (pyridine 3-aldehyde) and with D-glucuronate. In addition, the mechanism of enzyme inhibition by a number of 'aldose reductase' and aldehyde reductase inhibitors was studied and is reported.

## MATERIALS AND METHODS

### Materials

Sheep livers were obtained from the State Abattoirs, Homebush, N.S.W., Australia, and were transported to the laboratory in ice. Tissues were washed in 0.9% NaCl and stored at −20 °C until required.

$NADP^+$ and NADPH were purchased from Boeh-

Abbreviations used: ALR1 and ALR2, two major forms of NADPH-dependent aldehyde reductase.

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ringer Mannheim (Australia) Pty. (Sydney, N.S.W., Australia), *p*-carboxybenzaldehyde and L-gulono-$\gamma$-lactone were from Fluka (Buchs, Switzerland), 2-ethylhexanoic acid and *p*-nitroacetophenone were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and pyridine-3-methanol was from ICN Pharmaceuticals (Plainview, NY, U.S.A.). Alrestatin {AY22284; 1,3-dioxo-1*H*-benzo[*d*,*e*]isoquinoline-2(3*H*)-acetic acid} was kindly supplied by Dr. D. Dvornik (Ayerst Laboratories, Montreal, Quebec, Canada). Sorbinil [CP 45634; (+)-6-fluorospiro(chroman-4,4′-imidazoline)-2′,5′-dione] was a gift from Dr. R. Sarges (Pfizer Central Research, Groton, CT, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Distilled deionized water was used in all preparations and purifications.

Sephacryl S-200 and DEAE-Sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), Red A Matrex gel was obtained from Amicon (Australia) Pty. (Adelaide, S. Australia, Australia).

### Preparation of substrates and inhibitors

Aldehyde substrates that were insoluble in water were dissolved in 95% (v/v) ethanol. Alrestatin, sorbinil, valproate and 2-ethylhexanoate were suspended in water, and 0.1 M-NaOH in 1% (w/v) $NaHCO_3$ was added dropwise until the compound dissolved. The pH was then adjusted to 7.0 with 0.1 M-HCl. D-Glucuronate was purified by extraction with diethyl ether, followed by recrystallization from ethanol three times. This procedure removed a large amount of u.v.-absorbing material from the commercial preparation, decreasing the absorbance at 280 nm of a 1 M solution from 0.60 to 0.12. L-Gulonate was prepared from L-gulono-$\gamma$-lactone by the method of Moonsammy & Stewart (1967).

### Enzyme assay

Sheep liver ALR1 was assayed, at 30 °C, by measuring the change in absorbance at 340 nm as NADPH was oxidized. The assay mixture as a routine contained 100 mM-potassium phosphate buffer, pH 6.6, 100 $\mu$M-NADPH, aldehyde substrate and enzyme. These conditions were altered, on occasions, as specified in the text. Where ethanolic solutions of substrates were used, the resulting ethanol concentration in the assay was not inhibitory.

### Enzyme purification

All purification procedures were carried out at 0–4 °C, and all buffers contained 1 mM-2-mercaptoethanol. A 250 g portion of sheep liver was homogenized in 1 litre of 10 mM-potassium phosphate buffer, pH 7.0. The pH of the homogenate was lowered to 5.2 with 50% (v/v) acetic acid, and the solution was centrifuged at 13000 *g* for 75 min. The supernatant was filtered through glass-wool and the pH was adjusted to 7.0 with 10 M-KOH. The protein precipitated between 40% and 60% saturation with $(NH_4)_2SO_4$ was collected, dissolved in 10 mM-potassium phosphate buffer, pH 7.5 (buffer A), and dialysed overnight against 12 litres of buffer A. The dialysed solution was applied to a DEAE-Sepharose column (4.4 cm × 26 cm) that was equilibrated and eluted with buffer A. ALR1 was eluted as the second peak of *p*-nitrobenzaldehyde-reducing activity, behind the main bulk of the eluted protein, and was concentrated by precipitation by 75% saturation with $(NH_4)_2SO_4$. The precipitate was redissolved in approx. 16 ml of buffer A and dialysed overnight against 2 litres of the same buffer. The enzyme solution was then applied to a Red A Matrex gel column (1.0 cm × 17.5 cm) equilibrated with buffer A. The column was washed with 90 ml of this buffer, followed by a 200 ml linear gradient of 0–0.6 M-KCl in buffer A. Aldehyde-reducing fractions were combined and concentrated by ultrafiltration. The concentrated enzyme solution (2.5 ml) was applied to a Sephacryl S-200 column (3 cm × 73 cm) that was equilibrated and eluted with 50 mM-potassium phosphate buffer, pH 7.5. Active fractions were combined, concentrated by ultrafiltration and stored at −20 °C.

### Protein determinations

Protein concentrations were determined using Bradford's (1976) method, with bovine serum albumin as standard. Fractions obtained by column chromatography were continuously monitored for protein by recording the $A_{280}$.

### Fluorescence studies

Fluorescence studies were performed, at 25 °C, with a Perkin–Elmer MPF44B fluorescence spectrophotometer, equipped with a thermostatically controlled cell holder, and with the ratio photomultiplier (750–900 HV). A Perkin–Elmer DCSU-2 instrument was used to obtain corrected fluorescence spectra. The absorbance of solutions was kept below 0.3 at the wavelengths of emission (348 nm) and excitation (280 nm). Binding of ligands to sheep liver ALR1 was detected by measuring the accompanying change in intrinsic protein fluorescence. Suitable blank titration curves were carried out to account for any fluorescence due to minor impurities in the ligands. The molar fraction of enzyme bound at each concentration of ligand was determined (Luisi *et al.*, 1973), and dissociation constants were obtained from Klotz plots (Klotz *et al.*, 1946).

### Data analysis

Experimental data from initial-velocity studies in which the concentration of two substrates or a substrate–inhibitor pair were varied were computer-fitted to the appropriate rate equations by using the FORTRAN programs described by Cleland (1979) to obtain values for kinetic constants. All kinetic constants are expressed ± the standard error determined in the computer fit.

## RESULTS AND DISCUSSION

### Protection studies

Protection against inactivation by group-specific reagents were used to study binding of substrates and products to sheep liver ALR1. The thiol reagent 4,4′-dithiodipyridine gave 62% inhibition of the enzyme in 20 min at 30 °C, indicating the presence of thiol groups that are essential for full catalytic activity. NADPH provided good protection against the inhibition, but D-glucuronate actually enhanced the inhibition (Fig. 1). An essential arginine residue was also implicated by the inhibition of enzyme activity by the arginine-modifying reagent butane-2,3-dione. In this case substantial protection against inactivation was afforded by $NADP^+$ and L-gulonate (Fig. 1). These results suggest that the four

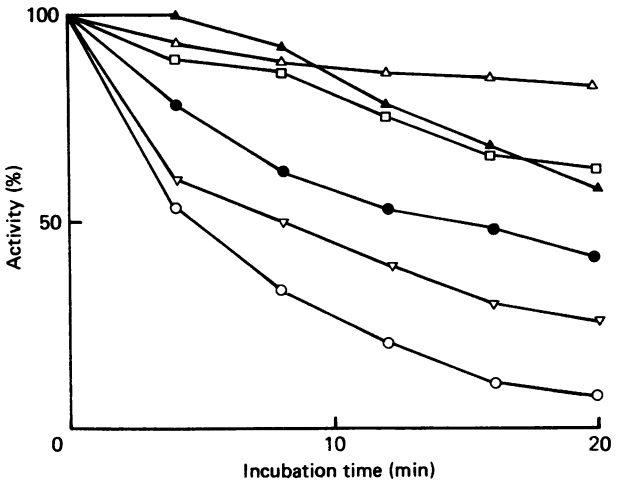



**Fig. 1. Inactivation of ALR1 and protection against inactivation**

The enzyme (1.5 $\mu$M) was incubated at 30 °C, and at the indicated times a sample was removed and enzyme activity determined in assay mixtures containing 100 mM-potassium phosphate buffer, pH 6.6, 10 mM-D-glucuronate and 100 $\mu$M-NADPH. (i) With 0.5 mM-dithiodipyridine in 100 mM-potassium phosphate buffer, pH 6.6, in the absence of substrates (●) or in the presence of 5 $\mu$M-NADPH (△) or 30 mM-D-glucuronate (○); (ii) with 50 mM-butane-2,3-dione in 50 mM-potassium borate buffer, pH 7.0, in the absence of substrates (▽) or in the presence of 50 mM-L-gulonate (□) or 1 mM-NADP$^+$ (▲). The Figure shows the activity of the inhibited enzyme expressed as a percentage of that of the enzyme incubated under the same conditions in the absence of inhibitor.

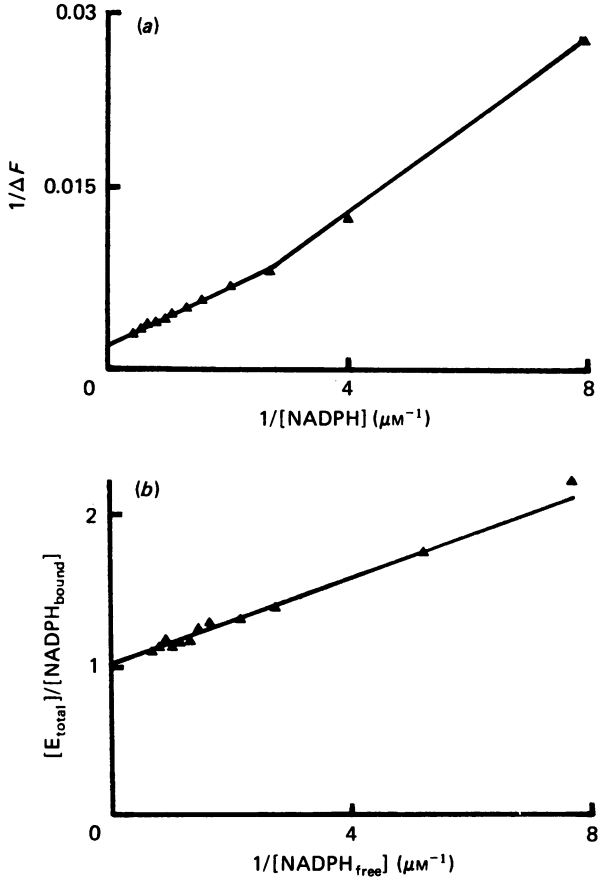



**Fig. 2. Effect of NADPH on the fluorescence of ALR1**

Enzyme (1.1 $\mu$M) in 50 mM-potassium phosphate buffer, pH 7.0, was titrated with NADPH and the change in fluorescence at 25 °C recorded. Fluorimeter settings were 280 nm excitation, 348 nm emission and 10 nm slit widths. (*a*) Double-reciprocal plot. $\Delta F = F_0 - F_t$, where $F_0$ is the enzyme fluorescence in the absence of NADPH and $F_t$ is the fluorescence at any subsequent point in the titration. (*b*) Klotz plot. Concentrations of free and bound NADPH were calculated from the values of $\Delta F$ and $\Delta F_{max.}$

substrates and products are all able to bind to the free enzyme.

Thermal-inactivation studies also provided evidence for binding of substrates and products to ALR1. When incubated at 41 °C for 25 min, 1 $\mu$M enzyme lost 65% of its activity. NADP$^+$ and NADPH provided good protection against this thermal inactivation, whereas D-glucuronate and L-gulonate enhanced the activity loss, again implying binding of these compounds to the free enzyme. In addition, recrystallized D-glucuronate enhanced activity loss to the same extent as the commercial glucuronate, indicating that this was not due to the presence of a contaminant in the glucuronate. In contrast, 30 mM-pyridine-3-aldehyde offered no protection against, or enhancement of, thermal inactivation, suggesting that this substrate did not bind to the free enzyme. However, the aldehyde reductase inhibitors phenobarbitone, alrestatin, sorbinil, 2-ethylhexanoate and valproate all provided various degrees of protection against thermal inactivation of ALR1 when present at saturating concentrations (45–50 times the $K_{ii}$ values reported in Table 4), indicating that these compounds are also able to bind to the free enzyme.

### Fluorescence studies

Intrinsic fluorescence was used to investigate substrate–enzyme interactions in sheep liver ALR1. The excitation and emission maxima were at 280 and 348 nm respectively. With excitation at 280 nm the enzyme fluorescence at 348 nm was quenched upon addition of D-glucuronate, NADP$^+$ or NADPH. In each case there was no significant shift of the emission maximum. This confirmed that these ligands bind to the free enzyme, and allowed the determination of dissociation constants. The enzyme was titrated with NADPH, and $\Delta F_{max.}$ was calculated from a double-reciprocal plot of fluorescence change versus NADPH concentration (Fig. 2*a*). The plot was not linear, indicating that the dissociation constant is low and comparable with the enzyme concentration used in the titration (Fujioka & Takata, 1981). $\Delta F_{max.}$ was used to calculate the fractions of bound and free ligand, and the data were then plotted as a Klotz plot (Fig. 2*b*). This gave values of $86.4 \pm 4.5\%$ for the maximum quench of enzyme fluorescence by NADPH and $0.110 \pm 0.017$ $\mu$M for the dissociation constant for NADPH (means from five titrations at different enzyme concentrations in the range 0.04–1.1 $\mu$M). A similar experimental protocol showed that NADP$^+$ caused an $80 \pm 1.4\%$ quench of enzyme fluorescence, and bound to the free enzyme with a dissociation constant of $3.68 \pm 0.40$ $\mu$M (means from three titrations). For D-glucuronate the maximal quench of enzyme fluorescence was $88.7 \pm 0.6\%$ and the dissociation constant was

**Table 1. Kinetic constants of sheep liver ALR1**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, enzyme and varied concentrations of aldehyde substrate and NADPH. The kinetic constants were obtained by computer-fitting data to the rate equation for a sequential mechanism (Cleland, 1979) and are expressed ± S.E.M.

| | *p*-Nitrobenzaldehyde as substrate | Pyridine 3-aldehyde as substrate | D-Glucuronate as substrate |
|---|---|---|---|
| $V$ | 3.6±0.2 μmol/min per mg | 6.0±0.6 μmol/min per mg | 3.9±0.6 μmol/min per mg |
| $K_m$ (NADPH) | 1.0±0.1 μM | 1.6±0.4 μM | 1.9±0.6 μM |
| $K_m$ (aldehyde) | 44±5 μM | 1.2±0.3 mM | 1.5±0.6 mM |
| $K_i$ (NADPH) | 1.0±0.2 μM | 1.4±0.1 μM | 0.51±0.19 μM |
| $K_i$ (aldehyde) | 44±1 μM | 1.0±0.3 mM | 0.39±0.18 mM |

**Table 2. Product inhibition of sheep liver ALR1 with pyridine-3-aldehyde as substrate**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, enzyme and varied concentrations of substrates and products as indicated. Inhibition constants were determined by computer-fitting the data to the appropriate rate equations (Cleland, 1979) and are expressed ± S.E.M.

| | | | | Inhibition constants | |
|---|---|---|---|---|---|
| Product | Varied substrate | Fixed substrate | Inhibition pattern | $K_{ii}$ | $K_{is}$ |
| $NADP^+$ | NADPH | 0.25 mM-Pyridine-3-aldehyde | Competitive | — | 16±1 μM |
| | Pyridine-3-aldehyde | 5 μM-NADPH | Non-competitive | 127±9 μM | 137±19 μM |
| Pyridine-3-methanol | NADPH | 0.25 mM-Pyridine-3-aldehyde | Non-competitive | 460±62 mM | 80±8 mM |
| | NADPH | 10 mM-Pyridine-3-aldehyde | Uncompetitive | 36±2 mM | — |
| | Pyridine-3-aldehyde | 5 μM-NADPH | Non-competitive | 88±4 mM | 285±48 mM |

**Table 3. Product inhibition of sheep liver ALR1 with D-glucuronate as aldehyde substrate**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, enzyme and various concentrations of substrates and products as indicated. The data were computer-fitted to the appropriate rate equations (Cleland, 1979) to determine kinetic constants, which are expressed ± S.E.M.

| | | | | Inhibition constants | |
|---|---|---|---|---|---|
| Inhibitor | Varied substrate | Fixed substrate | Inhibition pattern | $K_{ii}$ | $K_{is}$ |
| $NADP^+$ | NADPH | 1 mM-D-Glucuronate | Competitive | — | 19±1 μM |
| | | 10 mM-D-Glucuronate | Competitive | — | 6.5±0.3 μM |
| | D-Glucuronate | 5 μM-NADPH | Non-competitive | 86±5 μM | 142±14 μM |
| L-Gulonate | NADPH | 1 mM-D-Glucuronate | Non-competitive | 54±4 mM | 125±30 mM |
| | | 10 mM-D-Glucuronate | Uncompetitive | 35±2 mM | — |
| | D-Glucuronate | 5 μM-NADPH | Non-competitive | 64±6 mM | 83±18 mM |
| | | 50 μM-NADPH | Non-competitive | 33±4 mM | 59±16 mM |

57±8.5 mM (means from three titrations). Valproate also caused a large quench of enzyme fluorescence, indicating that it is able to bind to ALR1 to form a binary enzyme–inhibitor complex. The maximal fluorescence quench in this case was 75%, and the dissociation constant was 1.8 mM.

### Kinetic studies

Initial-velocity studies in which the concentrations of both NADPH and aldehyde substrate were varied indicate that sheep liver ALR1 follows a sequential mechanism with *p*-nitrobenzaldehyde or pyridine 3-aldehyde or D-glucuronate as substrate. Computer-fitting these data to the rate equation for a sequential mechanism (Cleland, 1979) gave the kinetic constants listed in Table 1.

A summary of the studies of product-inhibition by $NADP^+$ and pyridine-3-methanol with pyridine-3-aldehyde as substrate is shown in Table 2, and Table 3 summarizes the studies of product-inhibition by $NADP^+$ and L-gulonate with D-glucuronate as aldehyde substrate. These results are consistent with an ordered Bi Bi

mechanism in which NADPH binds to the free enzyme, followed by the aldehyde substrate. The alcohol product is released first, followed by $NADP^+$.

With *p*-nitrobenzaldehyde as substrate, inhibition by $NADP^+$ was competitive when NADPH was the varied substrate ($K_{is} = 21 \pm 1$ μM; [*p*-nitrobenzaldehyde] = 50 μM) and non-competitive when the concentration of *p*-nitrobenzaldehyde was varied ($K_{ii} = 95 \pm 8$ μM, $K_{is} = 150 \pm 13$ μM; [NADPH] = 5 μM). The other product, *p*-nitrobenzyl alcohol, increased the apparent reaction rate rather than inhibiting. $^1$H-n.m.r. spectra of the alcohol, after recrystallization from water, did not identify any impurities, although this purified alcohol also caused an apparent non-enzymic and complete oxidation of NADPH. A similar effect was not observed in the presence of *p*-nitrotoluene recrystallized from 50% (v/v) ethanol, indicating that the decrease of absorbance in the presence of *p*-nitrobenzyl alcohol is not due to reduction of the nitro group to a nitroso group. Although the nature of this non-enzymic reduction remains unknown, inexplicable blank rates in the presence of *p*-nitrobenzyl alcohol have been reported previously (Rivett & Tipton, 1981) and precluded the use of this compound as a product inhibitor. The results obtained for $NADP^+$ inhibition are consistent with a number of sequential mechanisms for sheep liver ALR1 where $NADP^+$ and NADPH bind to the free enzyme.

The binding of NADPH to ALR1 resulted in a 10 nm red-shift of the coenzyme absorption maximum from 336 to 346 nm. Difference spectra between free NADPH and NADPH in the presence of a fixed concentration of enzyme were recorded for increasing NADPH concentrations, and the differences between the absorbance changes at maximum (372 nm) and minimum (320 nm) wavelengths were determined. From a plot of absorbance change versus the ratio [NADPH]/[enzyme] a stoichiometry of 0.8 mol of NADPH/mol of enzyme was obtained by extrapolation. This is similar to the values of 0.86 and 0.85 reported for NADPH binding to the aldehyde reductases from pig kidney (Morpeth & Dickinson, 1980) and human liver (Wermuth *et al.*, 1977).

Further evidence for NADPH/$NADP^+$ binding to the free enzyme was provided by the protection afforded by $NADP^+$ and NADPH against enzyme inactivation due to the modification of thiol groups and arginine residues, the protection provided by $NADP^+$ and NADPH against thermal inactivation and the large quench of protein fluorescence seen upon addition of $NADP^+$ or NADPH to ALR1.

### High-substrate inhibition

Sheep liver ALR1 was inhibited by concentrations of D-glucuronate above 15 mM. This effect was not due to the large increase in ionic strength upon addition of high D-glucuronate concentrations, since the addition of KCl to equivalent ionic strengths did not inhibit the enzyme significantly. When the inhibition was studied at a number of NADPH concentrations the patterns of intersecting lines on both Dixon and Cornish-Bowden plots indicated that it was non-competitive (Fig. 3). The inhibition constants determined for recrystallized D-glucuronate and commercial D-glucuronate were 0.20 and 0.25 M respectively, suggesting that the inhibition was not due to the presence of an impurity in the D-glucuronate preparation. This inhibition would be

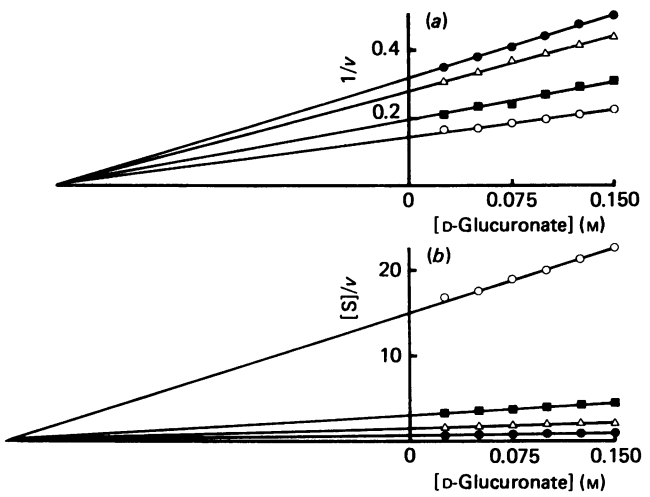



**Fig. 3. High-substrate inhibition of ALR1 by D-glucuronate**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, enzyme, D-glucuronate at concentrations indicated and NADPH concentrations of 2 μM (●), 5 μM (△), 15 μM (■) or 100 μM (○). (*a*) Dixon plot. (*b*). Cornish-Bowden plot. *v* is expressed as μmol/min per mg of protein.

explained by the binding of D-glucuronate to both the free enzyme and the E·$NADP^+$ complex at saturating concentrations. The formation of an E·Glucuronate binary complex was also implied by the large quench of enzyme fluorescence seen upon addition of D-glucuronate and by the enhancement of both enzyme inactivation by 4,4′-dithiodipyridine and thermal inactivation in the presence of D-glucuronate.

High concentrations of pyridine-3-aldehyde also inhibited sheep liver ALR1, with maximal activity at 7 mM-pyridine-3-aldehyde. The precise pattern of inhibition could not be determined in this case owing to the high absorbance of this compound at 340 nm, which also precluded the use of fluorescence techniques to study binding of pyridine-3-aldehyde to the enzyme. However, the lack of any effect by pyridine-3-aldehyde in studies of thermal inactivation and inactivation by modification of thiol groups suggests that this substrate may not bind to the free enzyme. Thus the mechanisms of inhibition by high concentrations of D-glucuronate and pyridine-3-aldehyde may differ, and it is possible that in the latter case only the $E\begin{matrix}\cdot NADP^+ \\ \cdot Aldehyde\end{matrix}$ dead-end complex may be formed.

The high-substrate inhibition of sheep liver ALR1 may be explained by proposing an anion-binding site on this enzyme, similar to that proposed for the pig liver enzyme (Branlant *et al.*, 1981), which binds substrates, such as D-glucuronate, containing a carboxy group. Substrates without this anion substituent (e.g. pyridine-3-aldehyde) bind to the E·$NADP^+$ complex, but are unable to bind to the free enzyme. To account for the observed patterns of product inhibition, it must be proposed that binding of aldehyde substrates to the free enzyme is non-productive. L-Gulonate also binds to the free enzyme, as evidenced by the enhancement of thermal inactivation and protection against inactivation by butane-2,3-dione, and may inhibit in a manner similar to the non-productive

**Table 4. Patterns of inhibition of ALR1 by 'aldose reductase' and aldehyde reductase inhibitors**

Inhibition patterns were determined in assay mixtures containing 100 mM-potassium phosphate buffer, pH 6.6, 20 μM-NADPH, inhibitor and either D-glucuronate or *p*-nitrobenzaldehyde as the varied substrate. Inhibition constants and patterns were obtained by computer-fitting the data to the appropriate rate equation (Cleland, 1979) and are expressed ± S.E.M.

| Inhibitor | Varied substrate | Inhibition pattern | Inhibition constants $K_{ii}$ | Inhibition constants $K_{is}$ |
|---|---|---|---|---|
| Phenobarbitone | D-Glucuronate | Non-competitive | 140 ± 12 μM | 203 ± 25 μM |
| | *p*-Nitrobenzaldehyde | Non-competitive | 58 ± 5 μM | 140 ± 21 μM |
| Sorbinil | D-Glucuronate | Uncompetitive | 1.20 ± 0.04 μM | — |
| | *p*-Nitrobenzaldehyde | Uncompetitive | 1.02 ± 0.03 μM | — |
| Alrestatin | D-Glucuronate | Uncompetitive | 26 ± 1 μM | — |
| | *p*-Nitrobenzaldehyde | Uncompetitive | 23 ± 1 μM | — |
| Valproate | D-Glucuronate | Uncompetitive | 22 ± 1 μM | — |
| | *p*-Nitrobenzaldehyde | Uncompetitive | 26 ± 1 μM | — |
| 2-Ethylhexanoate | D-Glucuronate | Uncompetitive | 50 ± 2 μM | — |
| | *p*-Nitrobenzaldehyde | Uncompetitive | 41 ± 2 μM | — |

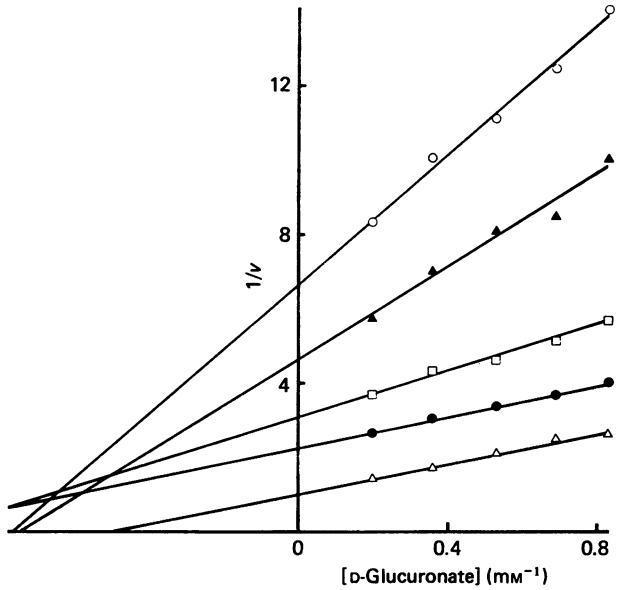



**Fig. 4. Inhibition of ALR1 by high valproate concentrations**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, 20 μM-NADPH, D-glucuronate at concentrations indicated and 0 μM- (△), 50 μM- (●), 100 μM- (□), 250 μM- (▲) or 500 μM- (○) valproate. *v* is expressed as μmol/min per mg of protein. For each valproate concentration the line of best fit was determined by linear regression.

binding of D-glucuronate. The formation of E·Alcohol complexes has been described previously (Wermuth & Von Wartburg, 1980; Morpeth & Dickinson, 1981; Magnien & Branlant, 1983), and, on the basis of product-inhibition patterns in the reverse direction, it was proposed that product release from the pig kidney and liver enzymes is partially random. There is no evidence for random release of products from sheep liver ARL1, although in this case it is not possible to study the reaction in the direction of alcohol oxidation.

**Aldehyde reductase inhibitors**

Sheep liver ALR1 was potently inhibited by a number of 'aldose reductase' and aldehyde reductase inhibitors. The pattern of inhibition by these compounds was determined with two aldehyde substrates (D-glucuronate and *p*-nitrobenzaldehyde), and the inhibition constants obtained by computer-fitting the data from double-reciprocal plots (in which the aldehyde concentration was varied at a number of inhibitor concentrations) to the appropriate rate equations are summarized in Table 4.

Phenobarbitone was a non-competitive inhibitor, indicating that this compound binds to the free enzyme as well as to one or both of the E·Coenzyme complexes. Sorbinil, alrestatin, valproate and 2-ethylhexanoate inhibited ALR1 uncompetitively, suggesting these inhibitors bind after D-glucuronate in the enzyme mechanism to form $E\cdot{}^{NADP^+}_{Inhibitor}$ dead-end complexes. In all the cases the pattern of inhibition and the inhibition constants were not markedly affected by changing the substrate.

At inhibitor concentrations greater than approx. 5–10 times the $K_{ii}$ values determined above for uncompetitive inhibition, the inhibition by sorbinil, valproate and 2-ethylhexanoate became non-competitive, resulting in non-linear secondary re-plots (Figs. 4 and 5). This is consistent with the formation of E·Inhibitor dead-end complexes as well as $E\cdot{}^{Coenzyme}_{Inhibitor}$ complexes when these compounds are present at elevated concentrations. The various degrees of protection against thermal inactivation by elevated concentrations of all five inhibitors provided further evidence for the formation of E·Inhibitor dead-end complexes, and fluorescence studies provided direct evidence for the formation of an E·Valproate dead-end complex. Although mixed inhibition of aldehyde reductase has been reported previously (Ris *et al.*, 1975; Whittle & Turner, 1978, 1981; Daly & Mantle, 1982), the failure of many workers to detect non-linear secondary re-plots may be due to the limited

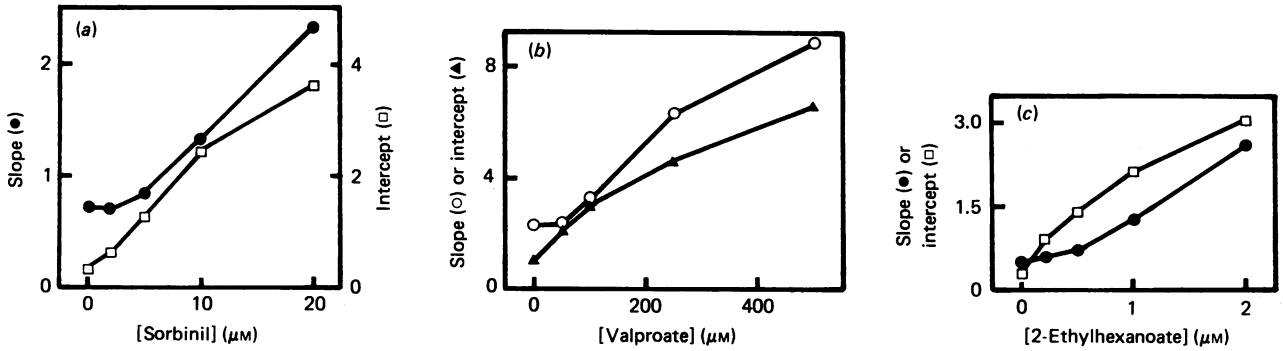
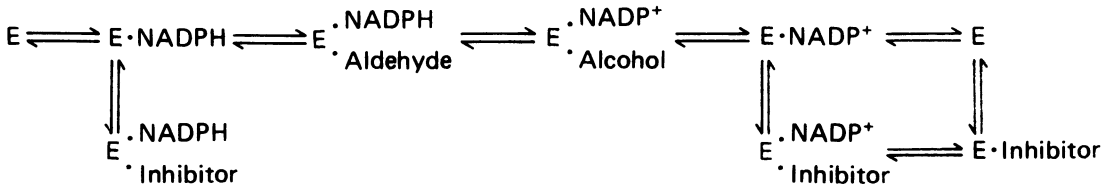



**Fig. 5. Inhibition of ALR1 by high inhibitor concentrations**

Assay mixtures contained 100 mM-potassium phosphate buffer, pH 6.6, 20 μM-NADPH, enzyme and various concentrations of D-glucuronate and (*a*) sorbinil, (*b*) valproate or (*c*) 2-ethylhexanoate. The data were plotted on double-reciprocal plots, and the line of best fit for each inhibitor concentration was determined by linear regression. The re-plots of the slopes and intercepts from this fit are shown.




**Scheme 1. Kinetic mechanism of ALR1**

The following points should be noted. 1. For inhibitors that are uncompetitive with respect to the aldehyde substrate, only the $E\cdot\frac{NADP^+}{Inhibitor}$ dead-end complex is required. 2. For inhibitors that are non-competitive with respect to the aldehyde substrate, the E·Inhibitor complex is formed. In addition, one or both of the dead-end ternary complexes $E\cdot\frac{Coenzyme}{Inhibitor}$ may also be formed. 3. In the case of high-substrate inhibition, it is proposed that only aldehyde substrates bearing a carboxy group may bind to the free enzyme, and such binding is unproductive. 4. Similarly, binding of the alcohol product to the free enzyme is also possible if it bears a carboxy group, but such binding is also unproductive.

range of inhibitor concentrations used. Inhibition of ox kidney aldehyde reductase by carboxylic acids involved binding of these inhibitors to both the free enzyme and the E·Coenzyme complexes (Daly & Mantle, 1982) at a site on the enzyme that is involved in binding inhibitors containing a carboxy group. Similarly, an 'anion-binding site' has been proposed for pig liver aldehyde reductase (Branlant *et al.*, 1981), and the observation that only the ionized forms of the inhibitors used in the present study affected sheep liver ALR1 (K. S. De Jongh, P. J. Schofield & M. R. Edwards, unpublished work) suggests the presence of a similar site on this latter enzyme.

In summary, the mechanism outlined in Scheme 1 accounts for the results of kinetic studies on sheep liver ALR1, as discussed above. For the ox kidney enzyme, Daly & Mantle (1982) reported a similar mechanism. However, because their initial-rate data did not conform to the predictions for a simple ordered mechanism, these authors favoured an ordered Bi Bi mechanism involving coenzyme-induced isomerization. A similar treatment of the initial-rate data for the sheep liver enzyme is not possible, since it requires the determination of kinetic constants in the reverse direction. Thus there is no evidence to suggest that coenzyme-induced isomerization of sheep liver ALR1 occurs in a similar manner.

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