

## The kinetic mechanism of the major form of ox kidney aldehyde reductase with D-glucuronic acid

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The steady-state kinetics of the major form of ox kidney aldehyde reductase with D-glucuronic acid have been determined at pH 7. Initial rate and product inhibition studies performed in both directions are consistent with a Di-Iso Ordered Bi Bi mechanism. The mechanism of inhibition by sodium valproate and benzoic acid is shown to involve flux through an alternative pathway.

Aldehyde reductase (EC 1.1.1.2) catalyses the reduction of a wide variety of aldehydes to the corresponding alcohol (Bachur, 1976). There are several forms of aldehyde reductase in most mammalian tissues (see e.g. Daly & Mantle, 1982) although only one major form has been reported in pig kidney (Flynn *et al.*, 1975; Branlant & Biellmann, 1980; Morpeth & Dickinson, 1980). The major form of aldehyde reductase catalyses the reduction of D-glucuronic acid to L-gulonic acid, a precursor of ascorbate (Flynn *et al.*, 1975; Chatterjee, 1973). It is therefore surprising that little work has been reported on the kinetic mechanism with this substrate, although Wermuth & von Wartburg (1980) have presented preliminary data for the human liver enzyme. Previous kinetic studies have used the model substrates 4-nitrobenzaldehyde (Bronaugh & Erwin, 1972), pyridine 3-aldehyde (Morpeth & Dickinson, 1981), glyceraldehyde (Toews, 1967; Davidson & Flynn, 1979; Morpeth & Dickinson, 1981) and succinic semialdehyde (Rivett & Tipton, 1981). Despite an early report that the mechanism was Ping Pong (Toews, 1967), most recent studies indicate that an ordered sequential mechanism is operative (Davidson & Flynn, 1979; Wermuth & von Wartburg, 1980; Morpeth & Dickinson, 1981; Rivett & Tipton, 1981) although Bronaugh & Erwin (1972) have reported a random sequential mechanism for ox brain aldehyde reductase. While current evidence suggests an ordered addition of substrates, the mechanism of product release has been described as ordered by Davidson & Flynn (1979) and Rivett & Tipton (1981) and random by Wermuth & von Wartburg (1980) and Morpeth & Dickinson (1981). Since there appears to be some uncertainty regarding the kinetic mechanism of aldehyde reductase and since it has been suggested that the mechanism may vary depending

on the substrate used (Morpeth & Dickinson, 1981; Branlant *et al.*, 1981) we have purified the major form of ox kidney aldehyde reductase (Daly & Mantle, 1982) and present here the results of a steady state kinetic study using a physiological substrate D-glucuronic acid.

### Materials and methods

#### Materials

NADPH and NADP<sup>+</sup> were purchased from Boehringer, D-glucuronic acid was supplied by Sigma, and L-gulonono- $\gamma$ -lactone was purchased from Fluka, Buchs, Switzerland. Sodium valproate was generously given by Reckitt & Coleman, Hull, U.K.

#### Preparation of aldehyde reductase (AR1)

The major form of ox kidney aldehyde reductase (AR1) was prepared as previously described (Daly & Mantle, 1982).

#### Preparation of L-gulonic acid

L-Gulonono- $\gamma$ -lactone (1M) was hydrolysed for 16 h in 1M-NaOH at 37°C. The solution was then adjusted to pH 7 with phosphoric acid. To ensure complete hydrolysis, the products were examined by t.l.c. on silica gel G plates in butan-1-ol/acetic acid/water (2:1:1, by vol.). After development, the plate was stained with 0.5% KMnO<sub>4</sub> in 1M-NaOH (Hay *et al.*, 1963).

#### Kinetic studies on AR1

Initial rate measurements were made by following the change in fluorescence at 460 nm following excitation at 345 nm with a Perkin-Elmer spectrofluorimeter MPF-44B connected to a Rikadenki chart recorder. The fluorimeter was calibrated over the range of NADPH concentrations used in the experiments (0.5–10  $\mu$ M) to ensure a linear response.

All solutions were filtered before use through glass fibre filters (Whatman GF-C) and were protected from dust at all times. The fluorimeter was calibrated each day with a known concentration of NADPH. All assays were performed at 30°C in 0.1M-sodium phosphate, pH 7. Assays were performed in duplicate and generally agreed within 5%.

All initial rate data were plotted in double-reciprocal form. The slopes ( $K_m/V$ ) and intercepts ( $1/V$ ) of each line were estimated by fitting the data to eqn. (1):

$$v = \frac{VS}{K_m + S} \quad (1)$$

by non-linear regression using the method of Wilkinson (1961).

Slope and intercept replots were fitted to straight lines by linear regression and the respective  $K_i$  values were obtained by extrapolating to the inhibitor concentration axis. Initial rate data was also fitted to eqn. (2):

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (2)$$

by non-linear regression (Cleland, 1967).

#### Determination of the equilibrium constant

The equilibrium constant was determined by the addition of enzyme to a mixture of all four reactants in 0.1M-sodium phosphate, pH 7. NADPH was held constant at 100  $\mu\text{M}$ , gulonic acid at 75 mM, glucuronic acid concentration was either 100  $\mu\text{M}$  or 200  $\mu\text{M}$  and NADP<sup>+</sup> was varied from 100 to 700  $\mu\text{M}$ . The change in NADPH concentration on going to equilibrium was measured by following the change in  $A_{340}$ . The concentration of the other reactants at equilibrium was calculated from the final measured concentration of NADPH.

#### Fitting of intercept replots for inhibition by various carboxylic acids

The data ( $y = \text{intercept}$ ;  $x = \text{carboxylic acid concentration}$ ) were fitted to eqn. (3) by linear regression and to eqns. (4) and (5) by non-linear regression.

$$y = ax + b \quad (3)$$

$$y = \frac{a + bx}{1 + cx} \quad (4)$$

$$y = \frac{a + bx + cx^2}{1 + dx} \quad (5)$$

Goodness of fit for each of these equations was determined by the criteria described by Mannervik *et al.* (1973).

## Results

### Kinetic studies

*Initial velocities.* When D-glucuronic acid was the variable substrate and NADPH was held constant at concentrations of 0.7–8  $\mu\text{M}$ , double reciprocal plots of initial velocity against D-glucuronic acid yielded a

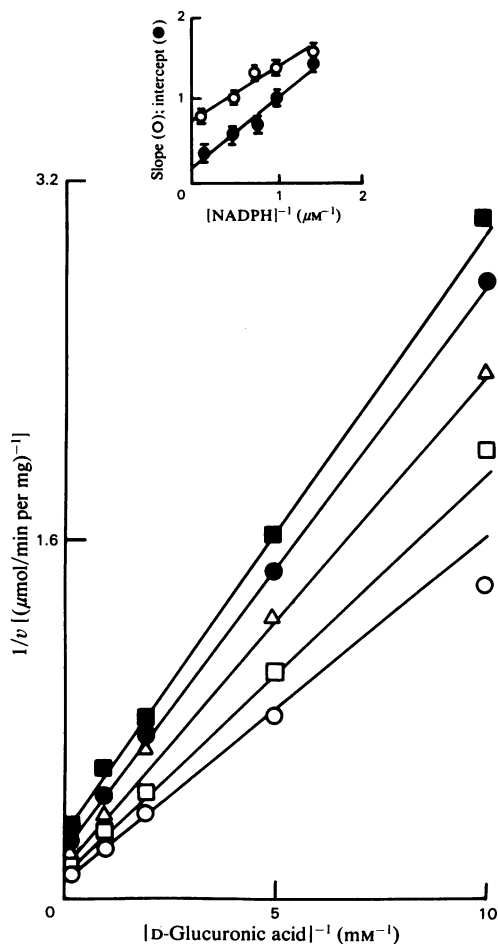


Fig. 1. Double reciprocal plots of initial velocity against D-glucuronic acid concentration at several NADPH concentrations

NADPH concentrations were 8.2  $\mu\text{M}$  (○), 2.05  $\mu\text{M}$  (□), 1.37  $\mu\text{M}$  (△), 1.025  $\mu\text{M}$  (●) and 0.685  $\mu\text{M}$  (■). All assays were performed with 10  $\mu\text{l}$  of a 1:50 dilution of purified AR1 (1.56 mg/ml) in a final assay volume of 2 ml. The replots of the slope and intercept values against the reciprocals of the NADPH concentrations are shown in the inset. For convenience the slope and intercept values are plotted in arbitrary units. The solid lines in the primary plots are based on kinetic coefficients estimated by fitting the data to eqn. (1) as described in the text. In the case of the replots the solid lines are regression lines.

family of straight lines intersecting in the upper left quadrant (Fig. 1). When NADPH was the variable substrate and D-glucuronic acid was held constant at concentrations of 0.1–5 mM, double reciprocal plots of initial velocity against NADPH again intersected in the upper left quadrant. The slopes and intercepts for each of these experiments were replotted against the reciprocal of the fixed substrate concentration. In each case the replots were linear (Fig. 1). These results suggest that a sequential mechanism is operating.

Initial velocity studies were also performed in the reverse direction. When L-gulonic acid was the variable substrate and NADPH was held constant at concentrations of 0.97–11.65  $\mu\text{M}$ , double reciprocal plots of initial velocity against L-gulonic acid intersected to the left of the vertical axis (Fig. 2). When NADPH was the variable substrate and L-gulonic acid was maintained at concentrations of 2.5–25 mM, double reciprocal plots intersected in the upper left quadrant. The slopes and intercepts of the lines from each experiment were replotted against the reciprocal of the fixed substrate concentration and were linear in each case (Fig. 2). These results are also in agreement with a sequential mechanism. Table 1 summarizes the kinetic constants for initial rate studies in both directions based on an ordered sequential mechanism, NADPH binding first.

**Product inhibition studies.** In the forward direction, product inhibition patterns were examined with L-gulonic acid and NADPH as inhibitors and with NADPH and D-glucuronic acid as variable substrates. When NADPH was the variable substrate, NADPH inhibition was competitive and L-gulonic acid inhibition was mixed. When D-glucuronic acid was the variable substrate, mixed inhibition patterns were obtained both with NADPH and L-gulonic acid (Fig. 3). Inhibition by L-gulonic acid was also examined at saturating levels of the non-varied substrate. When NADPH was saturating and D-glucuronic acid was varied, L-gulonic acid inhibition was mixed (Fig. 4); however, when D-glucuronic acid was saturating and NADPH was varied, L-gulonic acid inhibition appeared to be uncompetitive.

Product inhibition patterns were also examined in the reverse direction. It was not possible to examine D-glucuronic acid inhibition as, in the presence of

this compound, the reaction proceeded very rapidly to equilibrium and initial rates could not be measured. When NADPH inhibition was examined with NADPH as variable substrate, the inhibition pattern was competitive. When NADPH inhibition was examined with L-gulonic acid as variable substrate, a mixed pattern was obtained.

All replots for the product inhibition studies were linear. The inhibition constants calculated from the product inhibition experiments are summarized in Table 2.

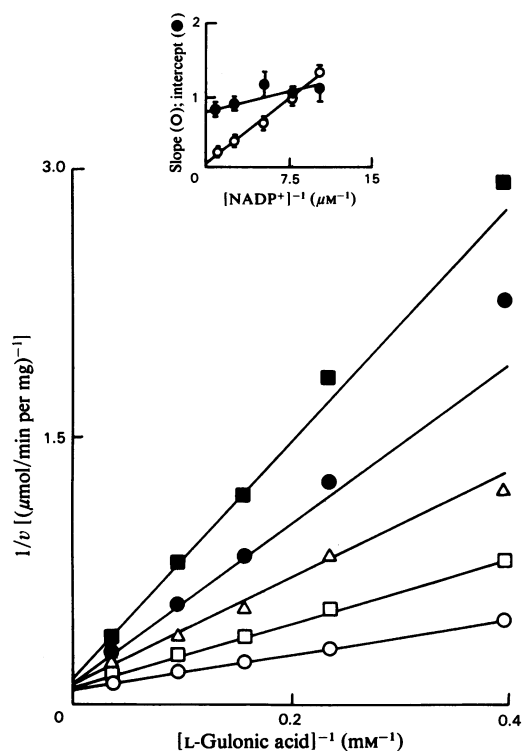


Fig. 2. Double reciprocal plots of initial velocity against L-gulonic acid concentration at several NADP<sup>+</sup> concentrations

For each assay, 5  $\mu\text{l}$  of purified AR1 (0.82 mg/ml) was used. The NADP<sup>+</sup> concentrations used were 11.65  $\mu\text{M}$  (○), 3.88  $\mu\text{M}$  (□), 1.94  $\mu\text{M}$  (△), 1.29  $\mu\text{M}$  (●) and 0.97  $\mu\text{M}$  (■).

Table 1. Kinetic constants of AR1  
All kinetic constants were determined by fitting the data to eqn. (2).

Substrate	$K_a$ ( $\mu\text{M}$ )	$K_{ia}$ ( $\mu\text{M}$ )	$K_p$ (mM)	$V_1$ ( $\mu\text{mol}/\text{min per mg}$ )
D-Glucuronic acid	$2.11 \pm 0.18$	$0.92 \pm 0.15$	$2.37 \pm 0.2$	$16.2 \pm 0.66$
Substrate	$K_q$ ( $\mu\text{M}$ )	$K_{iq}$ ( $\mu\text{M}$ )	$K_p$ (mM)	$V_2$ ( $\mu\text{mol}/\text{min per mg}$ )
L-Gulonic acid	$0.67 \pm 0.19$	$9.61 \pm 1.02$	$7.11 \pm 0.69$	$0.13 \pm 0.004$

**Inhibition by carboxylic acids.** The inhibition patterns obtained for AR1 in the forward direction in the presence of sodium valproate were investigated. Fig. 5 shows the results obtained when D-glucuronic acid was the variable substrate. A similar inhibition pattern was observed when NADPH was variable. The replots of Fig. 5 are shown in Fig. 6. The slope replot is linear but the intercept replot deviates from linearity at high concentrations of sodium valproate. Similar results were obtained when NADPH was the variable

substrate. Similar results were obtained with benzoic acid (results not shown). The intercept replot data was fitted to eqns. (3), (4) and (5) as described in the Materials and methods section. The residuals obtained by fitting the data to eqn. (5) generally appeared lower and more evenly spaced than those calculated for the other equations. This suggests that the intercept replots for benzoic acid and sodium valproate inhibition against either substrate are in the form of a 2/1 hyperbola rather than a straight line or a 1/1 hyperbola. The values obtained for the

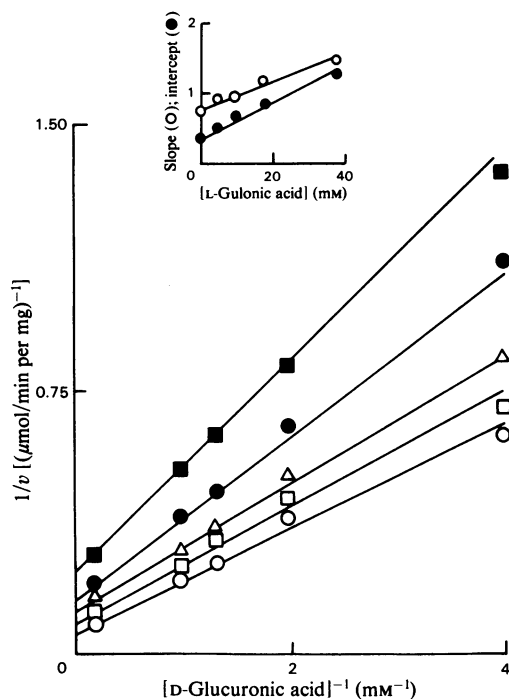


Fig. 3. L-Gulonic acid product inhibition with D-glucuronic acid as the variable substrate at a non-saturating concentration of NADPH

NADPH was held constant at  $10\ \mu\text{M}$ . For each assay,  $20\ \mu\text{l}$  of purified AR1 ( $0.82\ \text{mg/ml}$ ) was used. The L-gulonic acid concentrations were:  $0\ \text{(O)}$ ,  $4.7\ \text{mM}\ \text{(□)}$ ,  $9.4\ \text{mM}\ \text{(Δ)}$ ,  $18.75\ \text{mM}\ \text{(●)}$  and  $37.5\ \text{mM}\ \text{(■)}$ .

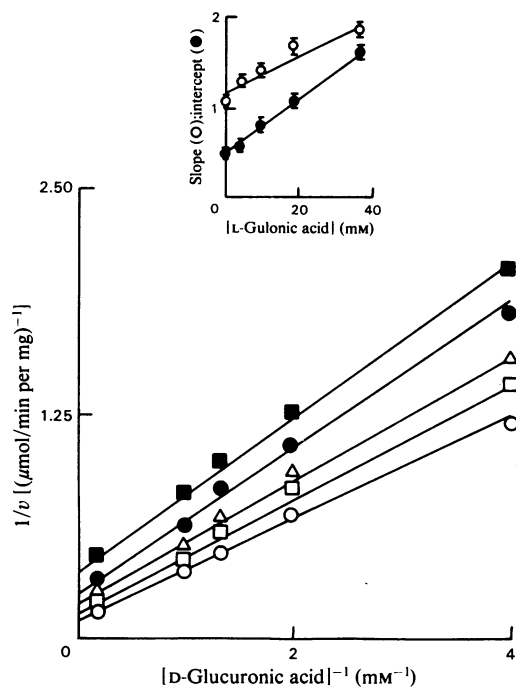


Fig. 4. L-Gulonic acid product inhibition with D-glucuronic acid as the variable substrate and NADPH at a saturating concentration

NADPH was held constant at  $74\ \mu\text{M}$ . For each assay,  $20\ \mu\text{l}$  of a 1:25 dilution of a stock solution of purified AR1 ( $1.56\ \text{mg/ml}$ ) was used. The L-gulonic acid concentrations were:  $0\ \text{(O)}$ ,  $4.7\ \text{mM}\ \text{(□)}$ ,  $9.4\ \text{mM}\ \text{(Δ)}$ ,  $18.75\ \text{mM}\ \text{(●)}$  and  $37.5\ \text{mM}\ \text{(■)}$ .

Table 2. Inhibition constants from product inhibition studies

Inhibitor	Variable substrate	$K_{is}$	$K_{ii}$
NADP <sup>+</sup>	NADPH	$16.0 \pm 5.0\ \mu\text{M}$	—
NADP <sup>+</sup>	Glucuronic acid	$104.68 \pm 7.33\ \mu\text{M}$	$87.49 \pm 10.50\ \mu\text{M}$
Gulonic acid	NADPH	$64.75 \pm 14.89\ \text{mM}$	$31.28 \pm 5.63\ \text{mM}$
Gulonic acid	Glucuronic acid	$39.0 \pm 4.68\ \text{mM}$	$17.40 \pm 2.0\ \text{mM}$
NADPH	NADP <sup>+</sup>	$0.2 \pm 0.07\ \mu\text{M}$	—
NADPH	Gulonic acid	$0.63 \pm 0.04\ \mu\text{M}$	$4.24 \pm 0.31\ \mu\text{M}$

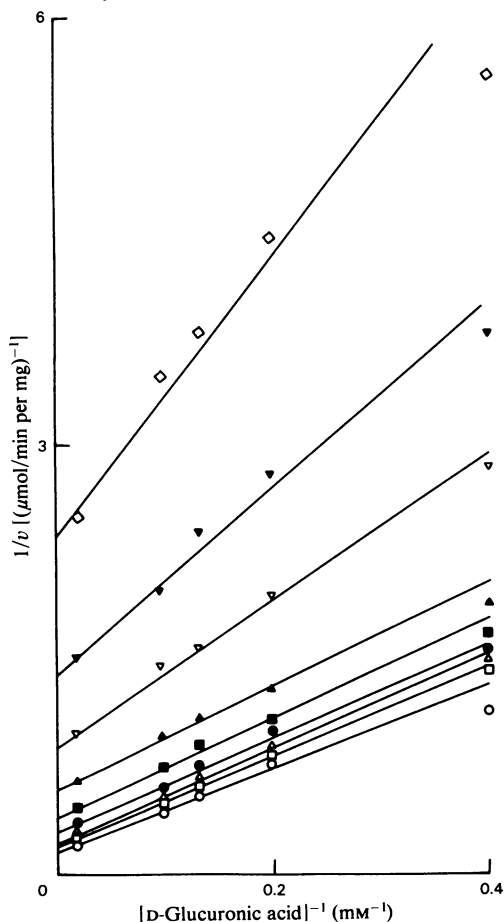


Fig. 5. Sodium valproate inhibition with D-glucuronic acid as the variable substrate

NADPH was held constant at 3 μM. For each assay, 10 μl of purified AR1 (0.82 mg/ml), diluted 1:25, was used. The sodium valproate concentrations were: 0 (○), 0.010 mM (□), 0.020 mM (△), 0.041 mM (●), 0.083 mM (■), 0.125 mM (▲), 0.250 mM (▽), 0.5 mM (▼) and 1.0 mM (◇).

constants *a*, *b*, *c* and *d* are summarized in Table 3. These constants, together with eqn. (5), were used to generate a theoretical curve for each data set. These

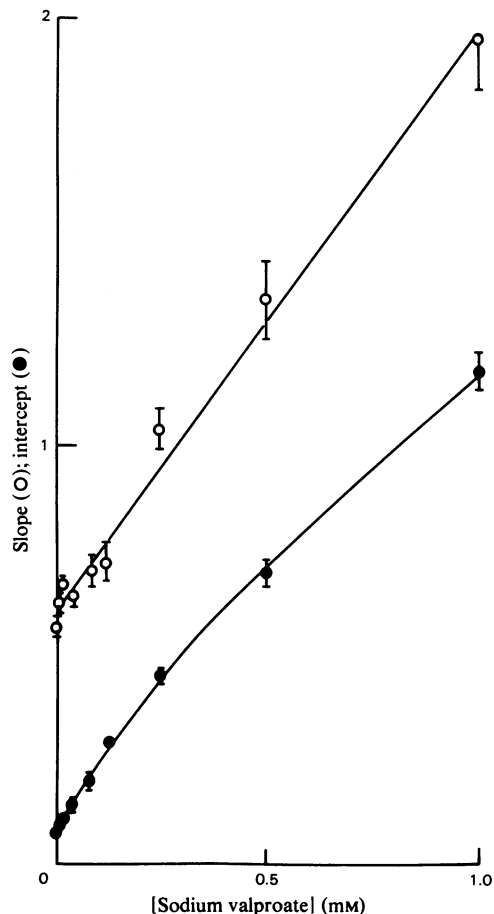


Fig. 6. Replots of slopes and intercepts from Fig. 5 versus sodium valproate concentration

The intercepts and slopes were fitted to eqns. 5 and 3 respectively, as described in the text.

Table 3. Values of the constants for a 2/1 hyperbola determined from the intercept replots of the carboxylic acid inhibition data

The values were obtained by fitting the intercept replot data to the equation for a 2/1 hyperbola:

$$y = \frac{a + bx + cx^2}{1 + dx}$$

using the program DEMGRID.

Inhibitor	Variable substrate	Value of:			
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
Benzoic acid	Glucuronic acid	$9.3 \times 10^{-2}$	$4.1 \times 10^{-1}$	$3.2 \times 10^{-2}$	$3.19 \times 10^{-1}$
Benzoic acid	NADPH	$1.25 \times 10^{-1}$	$6.3 \times 10^{-2}$	$2.0 \times 10^{-3}$	$9.9 \times 10^{-3}$
Sodium valproate	Glucuronic acid	$1.42 \times 10^{-1}$	$4.42 \times 10^{-3}$	$6.10 \times 10^{-6}$	$3.53 \times 10^{-3}$
Sodium valproate	NADPH	$1.69 \times 10^{-1}$	$6.66 \times 10^{-3}$	$3.02 \times 10^{-5}$	$1.87 \times 10^{-2}$

curves are shown for sodium valproate in Fig. 6, together with the experimental data.

### Equilibrium constant

The equilibrium constant of the reaction was determined to be  $(2.64 \pm 0.5) \times 10^{10} \text{ M}^{-1}$ .

### Discussion

The intersecting lines obtained from initial rate studies in both the forward and reverse directions are consistent with a sequential mechanism. The results obtained from product inhibition studies in the forward direction are consistent with either an ordered sequential mechanism where NADPH binds first or an Iso-Theorell–Chance mechanism with D-glucuronic acid as the first substrate to bind (Cleland, 1963). These two possibilities can be distinguished only by binding studies (Cleland, 1963). It has been shown that NADPH binds to aldehyde reductase from several sources (Flynn *et al.*, 1975; Wermuth *et al.*, 1977; Branlant & Biellmann, 1980; Morpeth & Dickinson, 1980) and we have evidence from  $^{31}\text{P}$ -n.m.r. experiments that NADP<sup>+</sup> binds to ox kidney AR1 (J. Feeney, A. Daly & T. J. Mantle, unpublished work).

There is some disagreement as to the order of product release for the major aldehyde reductase form, with Davidson & Flynn (1979) and Rivett & Tipton (1981) proposing an ordered release of products, whereas a random order of product release has been proposed by Wermuth & von Wartburg (1980). Morpeth & Dickinson (1981) have suggested that ordered product release occurs in the absence of products, but that a less efficient pathway of product dissociation occurs at high product concentrations. The results obtained in the present study for AR1 with D-glucuronic acid suggest that product release is ordered. A linear Dixon plot for L-gulonic acid inhibition was obtained (results not shown) suggesting that an alternative pathway of product release does not occur in this case. This conclusion is supported by the finding that linear mixed inhibition is observed even at high concentrations of L-gulonic acid when NADPH is saturating and D-glucuronic acid is the variable substrate.

It appears most likely, therefore, that AR1 follows an ordered sequential mechanism in both directions with the coenzyme binding first. For this mechanism the expressions 6 and 7 of Bloomfield *et al.* (1962a) and Dalziel (1957) respectively should be obeyed. [It should be noted that we have used Dalziel's original relationship, and when 7a and 7b are not an inequality the data is consistent with a Theorell–Chance mechanism.]

$$\frac{V_{\text{QR}}K_{\text{AB}}}{V_{\text{AB}}K_{\text{A}}K_{\text{B}}} < 1 \quad (6a) \quad \text{and} \quad \frac{V_{\text{AB}}K_{\text{QR}}}{V_{\text{QR}}V_{\text{Q}}V_{\text{R}}} < 1 \quad (6b)$$

$$\frac{\phi_{12}}{\phi_1\phi_2} \geq \frac{1}{\phi'_0} \quad (7a) \quad \text{and} \quad \frac{\phi'_{12}}{\phi'_1\phi'_2} \geq \frac{1}{\phi_0} \quad (7b)$$

Expressions 6a and 7a can be shown to be equivalent, as can 6b and 7b. In the nomenclature of Cleland (1963) expressions 6a and 7a equal:

$$\frac{V_2 K_a}{V_1 K_{\text{ia}}} < 1 \quad (8a)$$

and expressions 6b and 7b are equivalent to:

$$\frac{V_1 K_q}{V_2 K_{\text{iq}}} < 1 \quad (8b)$$

It has also been shown that for an ordered Bi Bi mechanism all the rate constants can be calculated, providing all the kinetic constants in both directions have been determined (Cleland, 1963).

For AR1:

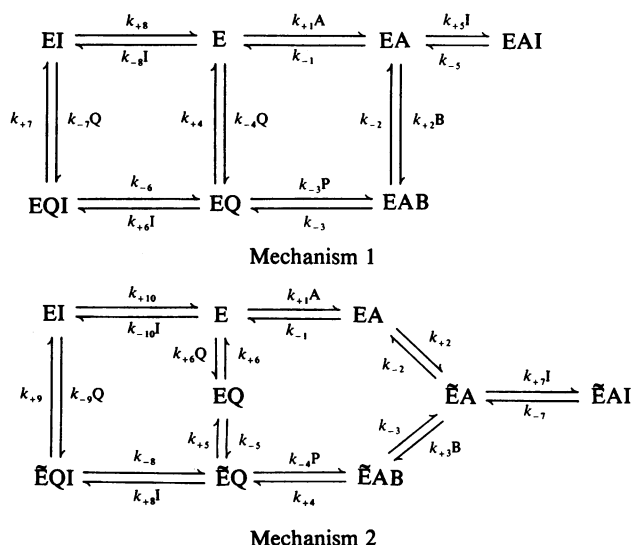
$$\frac{V_2 K_a}{V_1 K_{\text{ia}}} = 1.84 \times 10^{-2}$$

but

$$\frac{V_1 K_q}{V_2 K_{\text{iq}}} = 8.95$$

As the expression 8b does not hold, this suggests (Bloomfield *et al.*, 1962a; Dalziel, 1975) that either a random mechanism is operating or that an Ordered Bi Bi mechanism with isomerization of the binary complexes occurs (A similar finding has been reported for pig kidney aldehyde reductase with the D-glyceraldehyde/glycerol system (Morpeth & Dickinson, 1981). It should be noted that non-compliance with the Dalziel (1957) relationships could also be due to contamination of coenzyme preparations (Dalziel, 1963). In this case the expected relationships 8a and 8b should be observed on removal of the impurity. This would appear unlikely for AR1, since Morpeth & Dickinson (1981) have found that purification of NADP<sup>+</sup> had no effect on the kinetic parameters for pig kidney aldehyde reductase. Bloomfield *et al.* (1962b), using the kinetic data obtained by Nyggard & Theorell (1955) for yeast alcohol dehydrogenase, that of Weiner & Schwert (1958) for lactate dehydrogenase and that of Frieden (1959) for glutamate dehydrogenase, have suggested that these enzymes undergo coenzyme-induced isomerizations. More recent evidence of similar isomerizations has been obtained from X-ray diffraction studies of lactate dehydrogenase (Adams *et al.*, 1973) and of dihydrofolate reductase (Matthews *et al.*, 1978).

Carboxylic acid inhibition of the major aldehyde reductase form has been previously described and is known to give apparently non-linear replots (Ris *et al.*, 1975; Turner & Hick, 1975; Whittle & Turner,



Scheme 1. Possible mechanisms for AR1

1978). However, no detailed investigation of the kinetic mechanism has been reported. Since aldehydes which also contain carboxyl groups are very good substrates for the major aldehyde reductase form, it has been suggested that the active site contains a carboxyl binding site (Branlant & Biellmann, 1980). The presence of a second site involved in the binding of inhibitors containing carboxyl groups has been suggested by experiments involving the arginine modifying agents, phenylglyoxal and 4-carboxyphenylglyoxal (Branlant *et al.*, 1981). The present results, obtained with benzoic acid and sodium valproate, show that the intercept replots are in the form of a 2/1 hyperbola. Hyperbolic replots are typical of partial inhibition involving an alternative pathway (Cleland, 1970). Two possible mechanisms (1 and 2) of this type can be postulated for AR1, allowing for the fact that linear slope replots and 2/1 hyperbolic intercept replots are obtained when either substrate is varied. They are shown in Scheme 1. Rate equations, for the forward direction only, were derived for both mechanisms. Both Mechanisms 1 and 2 provide rate equations which predict the experimentally observed patterns for slope and intercept replots. The carboxylic acid inhibition results, therefore, cannot distinguish between an ordered Bi Bi mechanism involving coenzyme-induced isomerization (Mechanism 2) and a simple ordered sequential mechanism involving an alternative pathway through the ternary complex enzyme-NADP<sup>+</sup>-carboxylic acid (Mechanism 1). We favour Mechanism 2, since it is in agreement with the initial rate data which does not conform to the predictions for a simple ordered Bi Bi mechanism.

The inhibition of pig kidney aldehyde reductase by sodium barbitone has recently been proposed to follow a basically similar mechanism (Morpeth & Dickinson, 1981). Since carboxylic acids appear to bind to free enzyme and to the enzyme-coenzyme complex, it might be expected that substrates with a carboxyl group would behave similarly. No evidence for formation of D-complexes of this type has been obtained with D-glucuronic acid. This may be due to its rather bulky shape in comparison with the carboxylic acids used in the inhibition studies. We have evidence from kinetic studies with 4-carboxybenzaldehyde for an enzyme-NADP-4-carboxybenzaldehyde complex and an enzyme-4-carboxybenzaldehyde complex (D. M. Worrall, A. Daly & T. J. Mantle, unpublished work). A similar conclusion has recently been reported by Branlant *et al.* (1981).

Our results with D-glucuronic acid indicate that with this substrate a Di-Iso Ordered Bi Bi mechanism is operative. Since the details of this mechanism differ from those reported by groups working with other aldehyde substrates it is possible that the mechanism of the major form, particularly the order of product release, is substrate-dependent as suggested by Branlant *et al.* (1981). More detailed physical studies may provide further evidence for the postulated coenzyme-induced isomerization.

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