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Interactions of Uridine Diphosphate Glucose Dehydrogenase with the Inhibitor Uridine Diphosphate Xylose

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1. UDP-xylose and UDP-glucose both bind to UDP-glucose dehydrogenase in the absence of NAD^+, causing an enhancement of protein fluorescence. 2. The binding of UDP-xylose is pH-dependent, tighter binding being observed at pH8.2 than at pH8.7. 3. At low protein concentrations sigmoidal profiles of fluorescence enhancement are obtained on titration of the enzyme with UDP-xylose. As the protein concentration is increased the titration profiles become progressively more hyperbolic in shape. 4. The markedly different titration profiles obtained on titrating enzyme and the enzyme–NAD^+ complex with UDP-xylose suggests a conformational difference between these two species. 5. NAD^+ lowers the apparent affinity of the enzyme for UDP-xylose. 6. There is no change in the apparent molecular weight of UDP-glucose dehydrogenase on binding UDP-xylose. 7. Protein modification by either diethyl pyrocarbonate or 5,5′-dithiobis-(2-nitrobenzoate) does not 'desensitize' the enzyme with respect to the inhibition by UDP-xylose. 8. UDP-xylose lowers the affinity of the enzyme for NADH. 9. It is suggested that UDP-xylose is acting as a substrate analogue of UDP-glucose and causes protein-conformational changes on binding to the enzyme.

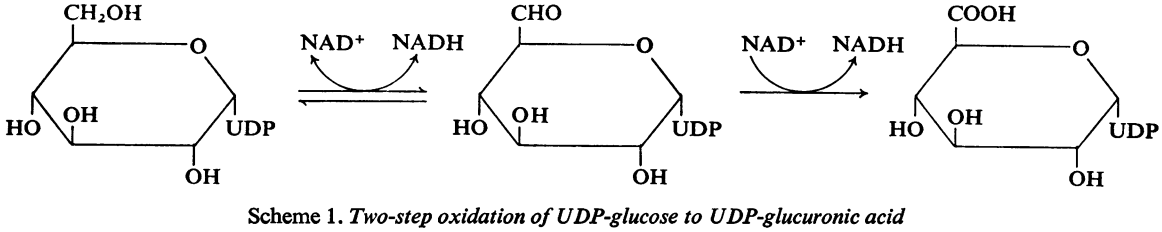
UDP-glucose dehydrogenase (EC 1.1.1.22) catalyses the four-electron oxidation of UDP-glucose to UDP-glucuronic acid. Nelsestuen & Kirkwood (1971) have postulated a two-step pathway (Scheme 1). Neufeld & Hall (1965) have shown the enzyme from various sources to be specifically and potently inhibited by UDP-xylose. The nature of the inhibition produced by UDP-xylose has been interpreted to suggest that UDP-xylose acts as an allosteric feedback inhibitor of UDP-glucose dehydrogenase. Similar conclusions have been reached for the enzyme from several other sources: hen oviduct (Bdolah & Feingold, 1968), micro-organisms (Ankel *et al.*, 1966), chick embryo (Darrow & Hendrickson, 1971), calf liver (Huang *et al.*, 1971), rat liver (Molz & Danishefsky, 1971; Sivaswami *et al.*, 1972), bovine nasal septum (Gainey & Phelps, 1972) and plants (Davies & Dickinson, 1972). Gainey *et al.* (1972) suggested that UDP-xylose was acting in addition as a substrate analogue, that is, as a competitive inhibitor. Some evidence for this contention has been provided by the binding studies carried out by Franzen *et al.* (1973).

In the present paper we further investigate in greater detail the inhibition of UDP-glucose dehydrogenase by UDP-xylose in order to gain a greater insight into the mechanism by which this enzyme is inhibited and possibly regulated.

Materials and Methods

Materials

Chemicals were obtained from the following: bovine serum albumin (fraction V), 2-mercaptoethanol and UDP-xylose from Sigma (London) Chemical Co., London S.W.6, U.K.; NAD^+, NADH and UDP-glucose from Boehringer Corp. (London) Ltd., London W.5, U.K.; Sephadex G-25 from Pharmacia, Uppsala, Sweden; diethyl pyrocarbonate and tryptophan from British Drug Houses Ltd., Poole, Dorset, U.K.; 5,5′-dithiobis-(2-nitrobenzoate) from Calbiochem (London) Ltd., London W.1,



Scheme 1. *Two-step oxidation of UDP-glucose to UDP-glucuronic acid*

U.K.; Norit A (acid washed) from Kodak Ltd., Kirkby, Lancs., U.K.

Bovine liver UDP-glucose dehydrogenase was purified to a specific activity of 3.35 units/mg of protein by using a modification of the method described by Zalitis & Feingold (1969). Polyacrylamide-gel electrophoresis of the purified enzyme showed the presence of a major protein peak corresponding to UDP-glucose dehydrogenase and a minor protein peak corresponding to about 5% impurity. No other dehydrogenase activity was present.

The purified enzyme was treated with Norit A in a similar way to that described by Taylor *et al.* (1948). Norit A was added to the enzyme solution to a concentration of 13 mg/ml. After gentle agitation for 10 min at 0–4°C the Norit A was removed by passage through a Millipore filter (pore size 0.3 μm). Some 90–100% of the enzymic activity was recovered after this procedure.

Methods

Enzyme assay. Unless otherwise stated the enzymic activity was assayed at 31°C by using a 1 ml 1 cm-light-path cuvette containing 100 μmol of glycine–NaOH buffer, pH 8.7, 1 μmol of NAD^+ and variable amounts of enzyme in a final volume of 1 ml. The reaction was started by the addition of 1 μmol of UDP-glucose and the increase in absorbance due to NADH production at 340 nm was followed in a Unicam SP.1800 double-beam spectrophotometer fitted with the appropriate blank. The sample cuvettes were brought to the temperature of measurement by preincubation for 2.5 min before the addition of UDP-glucose.

In the inhibition experiments with UDP-xylose the inhibitor was preincubated with the enzyme and NAD^+ before addition of UDP-glucose to start the reaction. Initial rates were recorded.

Protein fluorescence studies. The protein was titrated with UDP-xylose by using a titrating split-beam differential fluorimeter as described by Holbrook (1972). A split beam of monochromatic radiation (280 nm) was used to excite fluorescence in two 1 cm^2 quartz cuvettes, one containing UDP-glucose dehydrogenase and the other bovine serum albumin or tryptophan as a reference. Fluorescence was observed at 90° through Kodak Wratten no. 18A filters by using matched photomultipliers. In the differential mode the signals from each photomultiplier were subtracted by a small analogue computer (see Holbrook, 1972) and after calibrated amplification the difference signal was displayed as protein fluorescence on a chart recorder.

The fluorescence stopped-flow apparatus used was essentially that described by Bagshaw *et al.* (1972). Reactions were studied at room temperature (21 ± 2°C).

Modification of UDP-glucose dehydrogenase by 5,5′-dithiobis-(2-nitrobenzoate). About 0.3 nmol (Zalitis & Feingold, 1969) of enzyme (0.1 mg) was incubated with 1 μmol each of NAD^+ and UDP-glucose in a final volume of 1 ml of 0.1 M-Tris–HCl buffer, pH 8.0, at 35°C. NADH production was monitored by the increase in E_{340}. After about 10 s, 0.05 μmol of 5,5′-dithiobis-(2-nitrobenzoate) in 0.1 M-Tris–HCl buffer, pH 8.0, was added to the reaction cuvette. After about 2.5 min, when the rate of NADH production (ΔE_{340}) had markedly decreased, the reaction mixture was placed on ice. Excesses of NAD^+, NADH, UDP-glucose and 5,5′-dithiobis-(2-nitrobenzoate) were removed by passage of the reaction mixture through a Sephadex G-25 column (10 cm × 0.75 cm^2) previously equilibrated with 0.1 M-Tris–HCl buffer, pH 8.0. This enzyme was used in the inhibition studies with UDP-xylose.

Modification of UDP-glucose dehydrogenase with diethyl pyrocarbonate. Enzyme (0.6 mg) was treated with diethyl pyrocarbonate (34 μM) at 26°C in a final volume of 1 ml of 0.1 M-sodium phosphate buffer, pH 6.0. The diethyl pyrocarbonate was used as a freshly made solution in ethanol. The final concentration of ethanol in the reaction mixture was not higher than 1%.

Ethoxycarbonylation of histidine residues was followed at 242 nm in an SP. 1800 double-beam spectrophotometer fitted with quartz cuvettes and the appropriate blank. The number of histidine residues modified was estimated by using a value of 3200 litre·mol^{-1}·cm^{-1} for the molar extinction coefficient of *N*-imidazole ethoxycarbonylhistidine at 242 nm (Ovadi *et al.*, 1967). The modified protein was used in the activity and inhibition experiments.

Sedimentation studies. Sedimentation was carried out in a Spinco model E analytical ultracentrifuge in duplicate cells. The reference cell contained 0.7 ml of enzyme (8.0 mg/ml) in 0.1 M-Tris–HCl buffer, pH 8.0. The sample cell contained the same volume of enzyme plus 0.1 mM-UDP-xylose or a mixture of 0.1 mM-NAD^+ and 0.03 mM-UDP-xylose. The rotor speed was maintained at 59780 rev./min and the temperature at 20°C.

Results

Protein-fluorescence studies

The manual addition of 0.3 mM-UDP-xylose to the enzyme (0.33 μM-hexamer) alone at pH 8.1 resulted in a 5% enhancement of protein fluorescence. The addition of 1 mM-UDP-glucose to the enzyme under similar conditions gave an 8% enhancement of protein fluorescence. The continuous fluorimetric titration of the enzyme alone with UDP-xylose resulted in an enhancement of protein fluorescence (Fig. 1). The titration profile obtained at pH 8.1 is

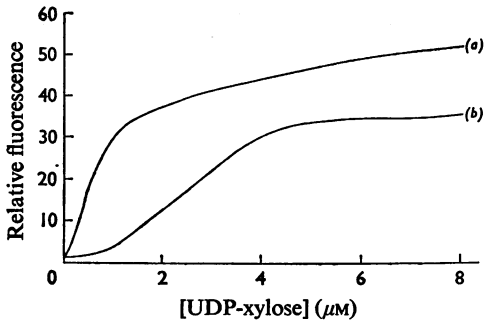


Fig. 1. *Continuous fluorimetric titration of UDP-glucose dehydrogenase with UDP-xylose*

Protein fluorescence was excited at 280nm. UDP-xylose (1mM) was continuously added (5μl/min) to a stirred solution of enzyme (10μg/ml) in 0.1M-glycine–NaOH buffer, pH8.1 (*a*) and pH8.7 (*b*). The temperature was 20°C. Signal amplification was ×10.

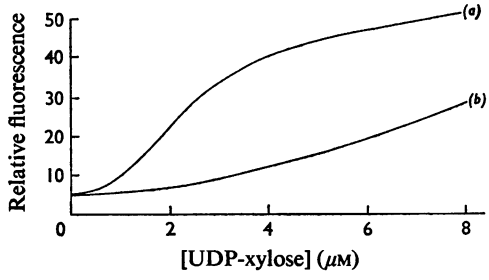


Fig. 2. *Protein-fluorescence enhancement on titration of the UDP-glucose dehydrogenase–NAD$^+$ binary complex with UDP-xylose*

Protein fluorescence was excited at 280nm. UDP-xylose (1mM) was continuously added (5μl/min) to a stirred solution of enzyme (10μg/ml), containing 0.045mM-NAD$^+$, in 0.1M-glycine–NaOH buffer, pH8.1 (*a*) and pH8.7 (*b*). Only part of the titration profile at pH8.7 is shown. The temperature was 20°C. Signal amplification was ×10.

markedly biphasic, whereas that obtained at pH8.7 is sigmoidal. The magnitude of the protein-fluorescence enhancement was pH-dependent. The concentration of UDP-xylose required to saturate the enzyme fully was greater at pH8.7 than at pH8.1, indicating a decrease in the affinity of the enzyme for UDP-xylose at the higher pH value.

A 6–8% enhancement of protein fluorescence was also observed on rapidly mixing UDP-glucose dehydrogenase in the fluorescence stopped-flow apparatus. The fluorescence-enhancement profiles were also biphasic in nature at pH8.1.

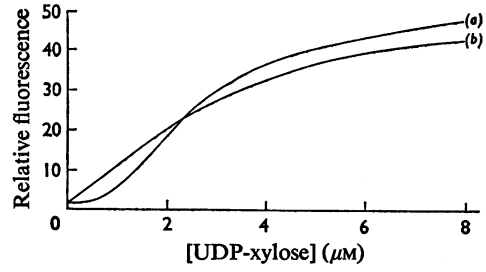


Fig. 3. *Continuous fluorimetric titration of various concentrations of UDP-glucose dehydrogenase with UDP-xylose*

Protein fluorescence was excited at 280nm. UDP-xylose was continuously added (5μl/min) to a stirred solution of enzyme, containing 0.045mM-NAD$^+$, in 0.1M-glycine–NaOH buffer, pH8.7. The concentration of enzyme used was (*a*) 0.01mg of protein/ml and (*b*) 0.1mg of protein/ml. The temperature was 20°C. Signal amplification was ×10.

The continuous fluorimetric titration of the enzyme–NAD$^+$ binary complex with UDP-xylose is shown in Fig. 2. The titration profiles obtained at pH8.7 and 8.1 were sigmoidal in shape. Again the magnitude of the enhancement was pH-dependent. At pH8.7 a 8–9% enhancement and at pH8.1 a 5–6% enhancement of protein fluorescence was obtained.

The shape of the titration profile obtained on titrating the enzyme–NAD$^+$ complex with UDP-xylose was dependent on the concentration of protein used. At relatively high protein concentrations (0.5mg/ml) only 30% of that protein-fluorescence enhancement measured at low protein concentration was observed. As the protein concentration was lowered the titration profile became progressively more sigmoidal in shape (Fig. 3). Conversely, as the protein concentration was increased the lag phase disappeared and the titration profile became progressively more hyperbolic in shape. At low protein concentration (0.025mg/ml or less) a further increase in the protein-fluorescence enhancement was observed after the addition of UDP-xylose had been stopped.

Modification studies with diethyl pyrocarbonate

Modification with diethyl pyrocarbonate was essentially complete after about 25min, and a total of 32 histidine residues per hexamer, corresponding to about 5 histidine residues per monomer, were found to have reacted. A difference spectrum between the diethyl pyrocarbonate-modified enzyme and the native enzyme showed a peak at 246nm characteristic of *N'*-ethoxycarbonylhistidine. The absence of a peak at 280nm (Mühlrad *et al.*, 1967) indicated the absence of *O*-ethoxycarbonylated tyrosine residues

in the enzyme. The modified enzyme retained 85% of the original enzyme activity when assayed soon after reaction with diethyl pyrocarbonate. After 24h, however, this value had fallen to about 70%. The native enzyme was 49% inhibited by 20μM-UDP-xylose, whereas the modified enzyme was 85% inhibited under similar conditions.

The enzyme, with 56 out of a total of 77 histidine residues (Gainey *et al.*, 1972) modified, was more than 95% inhibited by 50μM-UDP-xylose.

Incubation of the diethyl pyrocarbonate-modified enzyme with 0.6M-hydroxylamine in 0.1M-sodium phosphate buffer, pH7.0 (Melchior & Fahrney, 1970), resulted in a slow return (complete after about 100min) of about 90% of the original enzymic activity. A control experiment using modified enzyme and 0.1M-sodium phosphate buffer, pH7.0, alone showed no change in enzyme activity.

The kinetics of NADH production on the native enzyme in the presence of UDP-xylose at 26°C was characterized by an initial lag phase of up to 20s before maximum velocity was obtained. With the diethyl pyrocarbonate-modified enzyme this lag phase was further increased to 30–40s. Although the rate of NADH production decreased rapidly (owing to product inhibition) with time in the absence of UDP-xylose, such a marked decrease was not observed in the presence of UDP-xylose. This effect was the same for either the native or the modified enzyme.

Inhibition studies with 5,5′-dithiobis-(2-nitrobenzoate)-modified protein

The native protein was 76% inhibited by 50μM-UDP-xylose in the presence of saturating concentrations of NAD^+ and UDP-glucose (1mM). The same inhibition was observed whether the UDP-xylose was added before, or together with, UDP-glucose. The 5,5′-dithiobis-(2-nitrobenzoate)-modified enzyme was 73% inhibited under similar conditions. Some 95% of the original enzymic activity was recovered on the addition of 0.1mM-2-mercaptoethanol to the modified protein.

Ultracentrifugation studies

The enzyme, the binary enzyme–UDP-xylose and the tertiary enzyme–NAD^+–UDP-xylose complexes each had an $s_{20,w}$ of about 12S, corresponding to a molecular weight of approx. 300000.

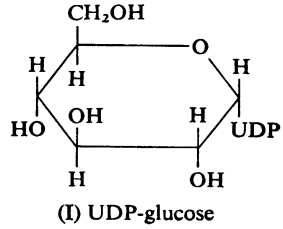
Discussion

Neufeld & Hall (1965) have shown UDP-xylose to be a potent and specific inhibitor of bovine liver and pea-seedling UDP-glucose dehydrogenase. The nature of the inhibition produced by UDP-xylose suggested that this compound was acting as an allosteric modifier of the enzyme. Similar conclusions have subsequently been drawn from the inhibition patterns obtained by using UDP-xylose and the enzyme from a number of different sources. Gainey *et al.* (1972) have suggested that UDP-xylose acts as an analogue of the substrate UDP-glucose, i.e. as a competitive inhibitor, with co-operative effects. The results of fluorimetric and equilibrium binding studies carried out by Franzen *et al.* (1973) may be interpreted as being consistent with this concept. Such a competitive mode of inhibition would be expected on comparing the structures of both the substrate (I) and the inhibitor (II).

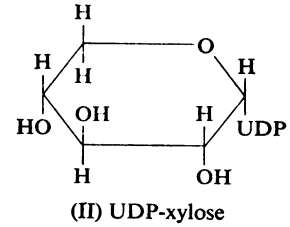
The essential difference between the substrate and the inhibitor is the absence of the bulky C-6 primary alcohol group on the UDP-xylose molecule.

Huang *et al.* (1971) have reported the inhibition of UDP-glucose dehydrogenase by various UDP-xylose analogues. The inhibition pattern produced by UDP-xylose at pH8.7 and 7.7 with respect to UDP-glucose and to NAD^+ may be taken as evidence that UDP-xylose binds at the same site as UDP-glucose. On lowering the pH value from 8.7 to 7.7 a tenfold decrease in the K_i value with respect to UDP-glucose was observed, whereas the K_i value with respect to NAD^+ remained the same at both pH values. If UDP-xylose is acting as an allosteric inhibitor then a change in the K_i value with respect to both UDP-glucose and NAD^+ might be expected.

Monod *et al.* (1963) have summarized some of the properties of allosteric proteins. Certain treatments, such as reaction with 5,5′-dithiobis-(2-nitrobenzoate), often 'desensitize' these proteins, that is they render them insensitive to inhibition by the feedback inhibitor. Modification of UDP-glucose dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoate)



(I) UDP-glucose



(II) UDP-xylose

or diethyl pyrocarbonate did not 'desensitize' the enzyme with respect to inhibition by UDP-xylose. In the case of modification by 5,5′-dithiobis-(2-nitrobenzoate) no change in the percentage inhibition by UDP-xylose was observed when either the native or the modified enzyme was used. With the diethyl pyrocarbonate-modified enzyme, however, the inhibition by UDP-xylose was actually increased twofold. Monod *et al.* (1963) also reported that in many cases the maximum inhibition that feedback inhibitors of allosteric proteins can achieve is less than total. An inhibition by competition for the same site(s) would give total inhibition. Such a total inhibition of UDP-glucose dehydrogenase activity is observed (Gainey *et al.*, 1972). J. Zalitis (personal communication) has shown that UDP-xylose binds at the same site as the thio-analogue of UDP-galactose.

Salitis & Oliver (1964) have shown UDP-galactose to be a competitive inhibitor of UDP-glucose dehydrogenase with respect to UDP-glucose. P. A. Gainey & C. F. Phelps (unpublished work) have shown that UDP-glucuronic acid, a competitive inhibitor of UDP-glucose dehydrogenase with respect to UDP-glucose, lowers the affinity of the enzyme for NADH, in a similar fashion to UDP-xylose. UDP-glucose and UDP-xylose both protect the two fast-reacting thiol groups of UDP-glucose dehydrogenase; further, the subsequent reaction profiles are superimposable (Gainey *et al.*, 1972). Bdolah & Feingold (1968) have reported UDP-xylose to be a non-co-operative competitive inhibitor of UDP-glucose dehydrogenase from *Aerobacter aerogenes*. Sivaswami *et al.* (1972) have shown UDP-xylose to be a competitive inhibitor of rat liver UDP-glucose at pH9.4, whereas at pH8.6 sigmoidal kinetics are observed. These observations suggest that UDP-xylose is capable of binding to UDP-glucose dehydrogenase at the UDP-glucose binding site. Although these results do not prove unequivocally the absence of allosteric UDP-xylose binding sites they are consistent with the concept that UDP-xylose is acting as a competitive inhibitor of the enzyme, competing for the UDP-glucose site. Allowing such a competitive mode of inhibition, UDP-xylose could still function as a potent and specific regulatory mechanism of the UDP-glucuronic acid pathway, as the results of Balduini *et al.* (1970), Gainey & Phelps (1972) and Castellani *et al.* (1967) suggest.

A comparison of the titration profiles obtained at pH8.1 and 8.7 (Figs. 1 and 2) shows that UDP-xylose binds much more tightly to the enzyme and to the enzyme–NAD^+ binary complex at the lower pH. This is in agreement with the studies of Huang *et al.* (1971) and Gainey *et al.* (1972), who have shown the inhibition of UDP-glucose dehydrogenase by UDP-xylose to be pH-dependent, the greater inhibition being observed at lower pH values.

Franzen *et al.* (1973) and Trayer & Trayer (1974) have shown that UDP-glucose can bind to the enzyme in the absence of NAD^+. Fig. 1 shows that UDP-xylose is also capable of binding to the enzyme in the absence of NAD^+.

The difference in the magnitude and shape of the protein-fluorescence enhancement observed at pH8.7 and 8.1 on titrating UDP-xylose into either the enzyme or the enzyme-NAD^+ complex (Figs. 1 and 2) may be explained by a pH-dependent conformational change of the protein.

Several workers have suggested an allosteric mode of inhibition by UDP-xylose as a result of the sigmoidal nature of the plots of percentage inhibition versus UDP-xylose concentration. Gainey *et al.* (1972) have suggested that UDP-xylose acts as a competitive inhibitor with co-operative effects. Results in the present paper offer an alternative interpretation of these findings. Fig. 2 shows the sigmoidal titration profile obtained by titrating UDP-xylose into a low concentration of the enzyme at pH8.1. Under normal steady-state assay conditions, that is, with low enzyme and high NAD^+ concentrations at pH8.7, the fluorescence titration profile would probably be highly sigmoidal. NAD^+ apparently lowers the affinity of the enzyme for UDP-xylose. If the addition of UDP-xylose is stopped during the fluorescence titration a subsequent increase in protein fluorescence enhancement is still observed. This would indicate that the profiles are a result of a kinetic, rather than an equilibrium, co-operative binding effect. Thus at the lower concentrations of UDP-xylose used in the steady-state inhibition studies, the full stoicheiometric inhibitory power of this compound is not realized, less inhibition is seen, and an apparent lag phase would result. Titration profiles that become progressively more hyperbolic in shape with increasing protein concentration are consistent with the above concept.

The protein-concentration effects (Fig. 3) might be explained by a classical dissociation–association system. Such a possibility is at present under investigation by using sedimentation techniques.

By assuming the cell water content of bovine liver to be approximately 50%, the physiological concentration of UDP-glucose dehydrogenase, calculated from the enzyme activity found initially in homogenates from 4kg of bovine liver (1750–3500 enzyme units), would be in the range of 0.25–0.5mg/ml. At this concentration of enzyme the titration profiles are not sigmoidal but hyperbolic in shape.

Gainey *et al.* (1972) have suggested the involvement of a thiol or an imidazole group in the binding of UDP-xylose to UDP-glucose dehydrogenase. Inhibition studies with the 5,5′-dithiobis-(2-nitrobenzoate)- and diethyl pyrocarbonate-modified enzyme indicate that neither a thiol nor histidine group(s) is involved in the binding of UDP-xylose.

The linear rate of NADH production observed by

using both native and modified enzyme in the presence of UDP-xylose is in agreement with the results of Gainey & Phelps (1974). They suggest that the presence of UDP-xylose decreases the affinity of the enzyme for NADH. The steady-state 'fall off' in NADH production is due to product inhibition (Zalitis & Feingold, 1968; Neufeld & Hall, 1965). Inhibition studies show that NADH (K_i approx. 10μM) is an order of magnitude greater in its inhibitory power than UDP-glucuronic acid (K_i greater than 100μM). Presumably, therefore, NADH is the major contributor to the product inhibition observed. In the presence of UDP-xylose the product inhibition by NADH is suppressed and the rate of NADH production becomes more linear.

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