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PARTIAL PURIFICATION AND PROPERTIES OF UDPG DEHYDROGENASE FROM *ESCHERICHIA COLI*

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SUMMARY

1. UDPG dehydrogenase (UDPG: NAD oxidoreductase, EC 1.1.1.22), was purified 88-fold to a specific activity of 2.3 from a derepressed strain of *Escherichia coli*.

2. The enzyme has a molecular weight of approximately 86 000 and a pH optimum of 9.0; K_m is 1.0 mM for UDPG and 0.05 mM for NAD, respectively.

3. UDPG, CDPGlc and dTDPGlc, but not GDPGlc or ADPGlc function as substrates. 3-Acetylpyridine adenine dinucleotide, 3-pyridinealdehydeadenine dinucleotide, thionicotinamide adenine dinucleotide, deamino adenine dinucleotide and 3-acetylpyridine deamino adenine dinucleotide, but not 3-pyridinealdehyde deaminoadenosine dinucleotide, are cosubstrates.

4. The enzyme is stable for 2 months when frozen at −4 °C in the presence of UDPG. UDPG and 2-mercaptoethanol are necessary for optimal enzyme activity.

5. UDPXyl is an inhibitor strictly competitive with UDPG and noncompetitive with NAD; NADH is an inhibitor strictly competitive with NAD and uncompetitive with UDPG. UDPglucuronate is an inhibitor competitive with UDPG at high UDPG concentrations; at low UDPG concentrations UDPglucuronate is an inhibitor which displays positive cooperativity.

INTRODUCTION

UDPG dehydrogenase (UDPG:NAD oxidoreductase, EC 1.1.1.22), which catalyzes the NAD-dependent conversion of UDPG to UDPglucuronate, has been purified to homogeneity from extracts of beef liver[1]. In addition, the enzyme has been purified partially from a number of eucaryotic sources, including hen oviduct[2], pea seedlings[3], and the yeast-like organism *Cryptococcus laurentii*[4]. Although UDPG dehydrogenase is doubtless widely distributed among procaryotes, to date the only bacterial UDPG dehydrogenase which has been more closely investigated is that from *Aerobacter aerogenes*. This enzyme was partially purified, however, neither highly active preparations nor good yields were obtained[5]. Recently, Lieberman *et al.*[6] reported

the presence of high levels of UDPG dehydrogenase in a mutant strain of *Escherichia coli*. In this paper the results of a study of the purification and properties of this enzyme are presented.

MATERIALS AND METHODS

Materials

UDPG, NAD, UDPXyl and UDPG analogs were purchased from the Sigma Chemical Co., St. Louis, Mo. NAD analogs were from P. L. Biochemicals, Inc., Milwaukee, Wis. UDP[U-^{14}C]glucose was from New England Nuclear Corp., Boston, Mass. and special enzyme grade $(NH_4)_2SO_4$ was from Schwarz/Mann, Orangeburg, N. Y. Venom phosphodiesterase (EC 3.1.4.1, Worthington Code:VPH), alkaline phosphatase (EC 3.1.3.1, Worthington Code: BAPC), deoxyribonuclease I (EC 3.1.4.5, Worthington Code: DP), and ribonuclease (EC 2.7.7.16, Worthington Code: R) were obtained from the Worthington Biochemical Corp., Freehold, N.J. All other reagents were of analytical grade.

Enzyme assay

UDPG dehydrogenase was assayed by following the reduction of NAD at 340 nm at 30 °C in a 1-cm light path cuvette in a Gilford Model 2000 Multiple Sample Absorbance Recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The reaction mixture contained 5.0 μmoles of UDPG, 0.5 μmole of NAD, and 0.1 μmole of glycylglycine buffer, pH 8.7, in a total volume of 1.0 ml. The reaction was initiated by addition of 2–20 μl of enzyme solution; since the reaction rate decreases rapidly with time, the initial velocity was determined during the first 15 s after mixing. A unit of enzyme activity is defined as the amount of enzyme required to produce 2 μmoles of NADH per min at 30 °C.

Paper chromatography

The following solvent systems were used for paper chromatography: Solvent I, ammonium acetate (0.5 M)–ethanol (95%) (7:3, v/v); Solvent II, *n*-propanol–ethyl acetate–water (7:1:2, v/v/v).

Protein concentration

Protein concentrations were determined as described by Lowry *et al.*[7].

Cell growth and enzyme purification

E. coli strain MC 153[8] (obtained from Dr A. Markovitz) was maintained at 4 °C on agar slants and grown on M-9 minimal medium supplemented as described by Markovitz *et al.*[9]. Cells were grown at 37 °C in a New Brunswick Microferm Laboratory Fermenter (New Brunswick Co., Inc., New Brunswick, N. J.). 9 l of medium were inoculated with 1 l of starter culture (300 Klett units, filter 42, determined with a Klett-Summerson Photoelectric Colorimeter, Klett Mfg. Co., N. Y.), and stirred at 200 rev./min with aeration at 12 lb/inch^2. When the culture reached the stationary phase (200–300 Klett units) the cells were harvested by centrifugation and washed with 0.05 M potassium phosphate buffer, pH 7.0, 0.01 M in 2-mercaptoethanol. 7 g of cells (wet wt) per l of medium were obtained. The cells could be stored frozen at

−10 °C for up to 1 week without appreciable loss of enzyme activity. All subsequent operations were performed at 0–4 °C. The term "buffer" refers to 0.05 M potassium phosphate; this and all other buffers used in the purification were 0.01 M in 2-mercaptoethanol.

Frozen cells (100 g) were suspended in 300 ml of buffer and stirred at 0–4 °C until a smooth paste resulted. The latter, in a 600-ml glass cooling cell, immersed in a bath at −3 °C, was sonicated continuously for 20 min at a setting of 9 A in a Branson Sonifier (Branson Instruments, Inc., Stamford, Conn.). During sonification, the temperature of the cell suspension varied between 5 and 9 °C. The supernatant fluid obtained by centrifugation of the sonicate at 27 000 × *g* for 20 min was treated with 3 mg each of ribonuclease and deoxyribonuclease per 100 ml of supernatant solution and held at 4 °C for 90 min. Solid $(NH_4)_2SO_4$ then was added to 30% saturation; 15 min after all the salt had dissolved, the precipitate was removed by centrifugation and the supernatant fluid was brought to 50% saturation with solid $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in a minimal volume of buffer (50–70 ml), and the solution was dialyzed for 18 h against 4 l of buffer, pH 7.0. The retentate was applied to a 6 cm × 17 cm column of DEAE-cellulose (Whatman DE-52; W. and R. Balston, Ltd., England) equilibrated with buffer, pH 7.0. The column was eluted with a linear gradient obtained with 2 l of 0.2 M potassium phosphate, pH 7.0, in the reservoir and an equal volume of buffer in the mixing vessel; the flow rate was 10 ml/min. UDPG dehydrogenase usually emerged in an 800-ml volume after 600 ml of eluate had been collected. The most active fractions (90% of the recoverable activity) were pooled and brought to 65% saturation with solid $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and dissolved in 14–18 ml of buffer, 0.005 M in UDPG. The above solution was applied to a Sephadex G-100 column (5 cm × 80 cm) equilibrated with 0.25 M potassium phosphate buffer, pH 7.0. The column was eluted with the same solution under a 20-cm water pressure head and fractions of 18 ml were collected at 25 min intervals. The enzyme emerged from the column after an initial 700 ml of eluate had been collected; active fractions were pooled (90% of total recoverable activity) and brought to 65% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 5 ml of buffer, 0.005 M in UDPG.

This solution was diluted 6-fold with 0.01 M 2-mercaptoethanol and applied to a 6 cm × 6 cm column of hydroxylapatite (Bio-gel HT, BioRad Laboratories, Richmond, Calif.) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, and packed on top of a 1-cm layer of Sephadex G-25 to increase the flow rate. The column was eluted with a linear gradient obtaiend with 2 l of 0.5 M potassium phosphate buffer, pH 7.0, in the reservoir and an equal volume of 0.01 M buffer in the mixing vessel; a flow rate of 80–100 ml/h was maintained with a 2-m pressure head. Fractions of 20 ml were collected; activity appeared in fractions emerging after the first 400 ml of effluent. Solid $(NH_4)_2SO_4$ was added to the pooled active fractions to 65% saturation and the precipitate was collected and dissolved in 5 ml of buffer, 0.005 M in UDPG. The purification is summarized in Table I.

Molecular weight determination by gel filtration chromatography

A 2.5 cm × 40.3 cm column of Sephadex G-150 packed in 0.25 M potassium phosphate buffer (pH 7.0), 0.01 M in 2-mercaptoethanol, was eluted under a pressure

TABLE I

PURIFICATION OF UDP-D-GLUCOSE DEHYDROGENASE FROM *E. coli*

	Volume (ml)	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	685	383	14 700	0.026	100
$(NH_4)_2SO_4$ fractionation	90	330	7 350	0.045	86
Diethylaminoethane cellulose fractionation	17	141	564	0.250	37
Sephadex G-100 fractionation	5	118	105	1.12	31
Hydroxylapatite fractionation	5	55	24	2.3	14

of 15 cm of water at 4 °C. The column was calibrated with the following standard proteins (see Fig. 1) supplied in the Pharmacia Calibration Kit (Pharmacia Fine Chemicals, Piscataway, N. J.). 1 = ribonuclease, 2 = chymotrypsinogen, 3 = ovalbumin, 4 = aldolase. In the figure $K_{av} = V_e - V_0/V_t - V_0$ where V_e = elution volume for a given protein, V_0 = void volume for the column (68.4 ml) and V_t = total column volume (203 ml). The sample of UDPG dehydrogenase was purified through the diethylaminoethane cellulose step and contained 10 enzyme units, all of which were recoverable from the Sephadex G-150 column.

Loss of enzyme activity and its subsequent restoration

In this experiment (see Fig. 3) a 17-fold dilution of the enzyme solution (spec. act. 1.2), purified through Sephadex G-100, was prepared in 0.05 M potassium phosphate buffer (pH 7.0) 0.01 M in 2-mercaptoethanol and 5 mM in UDPG. Three aliquots of this dilute solution were then removed. Aliquot one was stored for 48 h at 0–4 °C. At this time there was only a 5% loss of enzyme activity. A second aliquot (5.0 ml) was dialyzed at 0–4 °C *versus* 2 l of 0.05 M potassium phosphate buffer (pH 7.0), 0.01 M in 2-mercaptoethanol, for 7 h and then *versus* another 2 l of the same buffer for an additional 17 h. The retentate was then assayed for enzymic activity. At this time one portion of the retentate was stored at 0–4 °C (control) and another portion was made 5 mM with respect to UDPG and also stored at 0–4 °C. The solid symbols (▲ and ■) in the figure represent the change in enzyme activity with time of these enzyme preparations. A third aliquot (5.0 ml) of the initial enzyme dilution was also dialyzed as described above *versus* buffer without 2-mercaptoethanol. After 24 h of dialysis the retentate was assayed for enzyme activity and was then divided into four aliquots. One aliquot received no addition (control), ◇; a second aliquot was made 5 mM with respect to UDPG (+UDPG), □; the third aliquot was made 0.01 M with respect to 2-mercaptoethanol (+ME), △ and the fourth aliquot was made 5 mM with respect to UDPG and 0.01 M with respect to 2-mercaptoethanol (+ME, +UDPG), ○. All mixtures were maintained at 0–4 °C for an additional 24 h, during which time they were periodically assayed for enzyme activity. The results of these assays are shown by open symbols.

RESULTS AND DISCUSSION

Product identification

The enzyme, purified through the Sephadex G-100 step, was incubated for 1 h with UDP[U-^{14}C]glucose and NAD in 0.1 M glycylglycine buffer, pH 8.7. The entire reaction mixture was subjected to paper chromatography on Whatman 3 MM paper (W. and R. Balston, Ltd) in Solvent I for 17 h; two major radioactive areas were obtained with relative intensities of 0.2 and 0.8 in that order from the origin. These correspond to the positions for UDPglucuronate and UDPG, respectively, and indicate 20% conversion of UDPG to UDPglucuronate. A fluorescent area with the mobility of NADH was also present.

The radioactive material corresponding to UDPglucuronate was purified by rechromatography in Solvent I, desalted by irrigation for 72 h with Solvent II, eluted, mixed with unlabeled UDPglucuronate, and rechromatographed in Solvent I. A single radioactive area was present which co-chromatographed with authentic UDPglucuronate. An aliquot of radioactive material was mixed with unlabeled UDPglucuronate and subjected to hydrolysis in 0.1 M HCl for 30 min at 100 °C. Paper chromatography of the hydrolysate in Solvent II separated two radioactive compounds which coincided with D-glucuronic acid and D-glucuronolactone. Treatment with phosphodiesterase and alkaline phosphatase of an aliquot of the radioactive material with the chromatographic mobility of UDPglucuronate resulted in liberation of one major radioactive substance which co-chromatographed with carrier D-glucuronic acid in Solvent II. The reaction of another aliquot with UDPglucuronate carboxy-lyase (EC 4.1.1.35) from *Cryptococcus laurentii* (prepared to Step 3 according to Ankel and Feingold[10]) resulted in the quantitative formation of a single new radioactive substance which co-chromatographed with UDPXyl in Solvent I. Either acid or enzymic hydrolysis of this new radioactive material resulted in the appearance of a single radioactive component which co-chromatographed with D-xylose in Solvent II. The above results show that UDPglucuronate is the product of the NAD-linked dehydrogenation of UDPG catalyzed by the *E. coli* enzyme preparation.

The area corresponding to unreacted UDPG was also eluted from the chromatogram, hydrolyzed in 0.1 M HCl for 30 min, and the free sugars present in the hydrolysate were examined by gas–liquid chromatography. To achieve this, the hydrolysate was dried and reacted with the silylation mixture described by Sweeley *et al.*[11]. Gas chromatographic analysis revealed the presence of glucose and the possible presence of galactose in the relative proportions 15:1, respectively.

Substrate and cosubstrate analog studies

A number of UDPG analogs, all at concentrations of 5 mM, were assayed for their ability to react with the enzyme under otherwise usual assay conditions. Reaction rates relative to that with UDPG were: UDPG, 100%; CDPGlc, 5.5%; GDPGlc, 0%; dTDPGlc, 16.7%; ADPGlc, 0%. A number of NAD analogs were also assayed for their ability to react with the enzyme. These analogs replaced NAD at concentrations of 0.5 mM under otherwise usual assay conditions; changes in absorbance were recorded at the appropriate wavelengths for the reduced forms (P. L. Biochemicals, Inc., Milwaukee, Wisc.). Reaction rates relative to that with NAD were: NAD, 100%; 3-acetylpyridine adenine dinucleotide, 186%; 3-pyridinealdehyde adenine

dinucleotide, 13%; thionicotinamide adenine dinucleotide, 11%; *N*-deaminoadenosine dinucleotide, 76%; 3-acetylpyridine deamino adenosine dinucleotide, 94%; 3-pyridinealdehyde deamino adenosine dinucleotide, 0%.

Michaelis constants and linearity of the enzyme assay

K_m values calculated according to Lineweaver and Burk[12] for UDPG and NAD were 1.0 mM and 0.05 mM, respectively (see Figs 4–8). The initial velocity of the enzymic reaction was directly proportional to the amount of added enzyme up to a change of 0.30 absorbance unit/min. These determinations were all carried out with enzyme purified through the Sephadex G-100 step.

Molecular weight

The molecular weight is 86 000 as determined by gel filtration (Fig. 1).

pH optimum

The optimum pH for the dehydrogenation was determined using 0.1 M potassium phosphate buffer between pH 6.4 and 7.6 and 0.1 M glycine–NaOH between pH 8.2 and 9.8. 20 μg of protein (enzyme spec. act. 2.0) were used for each assay. The optimum pH is 9.0 as shown in Fig. 2.

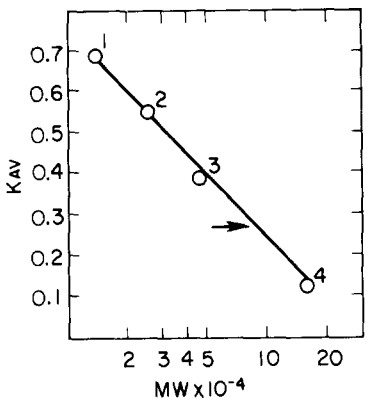


Fig. 1. Molecular weight determination by gel filtration chromatography. The arrow in the figure represents the K_{av} for UDPG dehydrogenase. Other details are given in Materials and Methods.

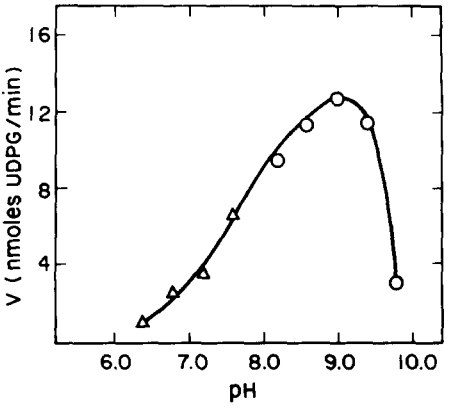


Fig. 2. The pH optimum for the enzymic reaction. Enzyme assay conditions are as described in Materials and Methods except that pH and buffer were varied as described in the text.

Enzyme purity

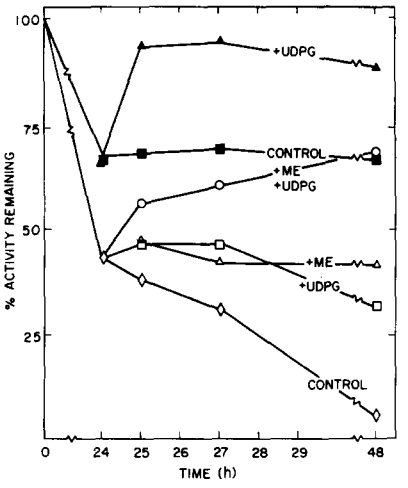
At present it is not possible to asses the purity of the various enzyme preparations since none of the protein bands demonstrated by polyacrylamide disc gel electrophoresis can be stained for enzymic activity.

Enzyme stability

The various preparations obtained during the purification procedure are relatively stable when kept frozen at −5 °C in the presence of 0.05 M potassium phos-

phate buffer (pH 7.0), 0.01 M in 2-mercaptoethanol. The stability is further enhanced in 5 mM UDPG. For example, an enzyme preparation from the hydroxylapatite step (4.8 mg/ml of protein) lost none of its activity after 8 weeks when stored frozen at −5 °C with UDPG and 50% of its activity when stored in the absence of UDPG.

The experiments described by Fig. 3 indicate that 2-mercaptoethanol as well as UDPG are necessary for maximum enzyme activity and that their effect is additive. These results suggest that UDPG and 2-mercaptoethanol have different functions in maintaining enzyme activity. While 2-mercaptoethanol doubtless functions mainly to keep sulfhydryl groups reduced, the role of UDPG is probably conformational. Such an effect of UDPG in maintaining maximal enzyme activity has not been previously demonstrated for the other UDPG dehydrogenases investigated and might offer the key to their stability during isolation.



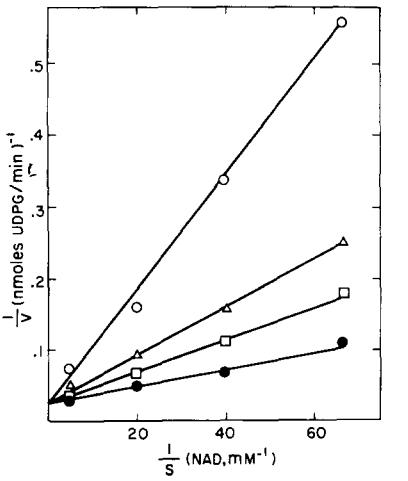


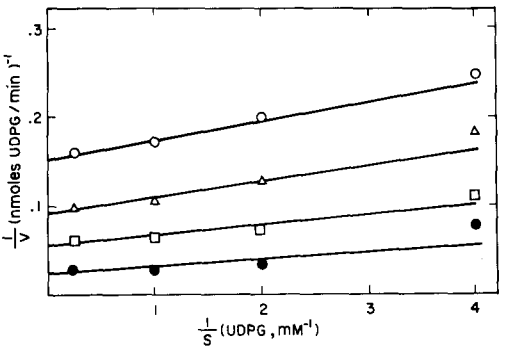
Fig. 3. Loss of enzyme activity and its subsequent restoration. The details of this experiment are presented in Materials and Methods. ME, mercaptoethanol.

Fig. 4. Inhibition of UDPG dehydrogenase by NADH at various concentrations of NAD. Standard assay conditions were used, except that the NAD concentration was varied as shown, and NADH was included in the assay medium at the following concentrations (μM): 0, ●; 15, □; 40, △; 75, ○. 60 μg of protein (enzyme spec. act. 1.2) was used for each assay, which was run in duplicate.

Inhibition studies

The effect of UDPglucuronate, NADH, and UDPXyl on enzyme activity is illustrated in Figs 4 through 8. Inhibition constants were calculated in the usual way[13,14] by plotting the reciprocal of the initial velocity ($1/v$) *versus* the inhibitor concentration at various constant substrate values. Fig. 4 indicates that NADH is an inhibitor competitive with NAD ($K_i = 25\ \mu M$) while Fig. 5 indicates that NADH is an inhibitor uncompetitive with UDPG ($K_i = 40\ \mu M$).

Inhibition of UDPG dehydrogenase by UDPXyl is non-competitive with respect to NAD (Fig. 6, $K_i = 250\ \mu M$) and competitive with respect to UDPG (Fig. 7, $K_i = 27\ \mu M$). These results are in contrast to findings with UDPG dehydrogenase from beef liver[16], mature hen oviduct[2], embryonic chick liver and eye[15], pea seedlings[3], and *C. laurentii*[4]. With the enzyme from each of these sources UDPXyl has been



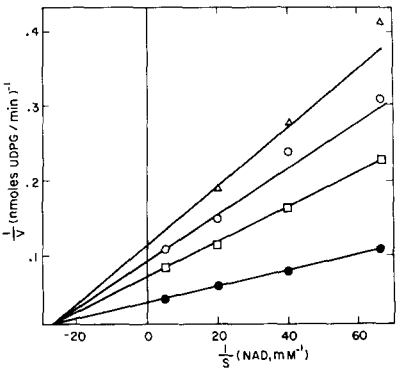


Fig. 5. Inhibition of UDPG dehydrogenase by NADH at various concentrations of UDPG. Standard conditions of assay were used except that the UDPG concentration was varied as shown and NADH was included in the assay medium at the following concentrations (μM): 0, ●; 100, □; 200, △; 300, ○. 60 μg of protein (enzyme spec. act. 1.2) was used for each assay, which was run in duplicate.

Fig. 6. Inhibition of UDPG dehydrogenase by UDPXyl at various concentrations of NAD. Standard conditions of assay were used except that the NAD concentration was varied as shown and UDPXyl was included in the assay medium at the following concentrations (μM): 0, ●; 250, □; 400, ○; 600, △. 60 μg of protein (enzyme spec. act. 1.2) was used for each assay, which was run in duplicate.

shown to be a highly specific inhibitor which exhibits varying degrees of positive cooperativity. Neufeld and Hall[16] originally suggested that UDPXyl acts as a specific feedback inhibitor of UDPG dehydrogenase in those tissues in which UDPXyl is a donor of the D-xylosyl moiety. In *A. aerogenes*, on the other hand, which contains no D-xylose, UDPXyl does not function as a cooperative type inhibitor[5]. The simple non-cooperative inhibitory effect of UDPXyl on the *E. coli* enzyme suggests that in this organism also UDPXyl does not function as donor of the D-xylosyl moiety and that D-xylose probably is not present in the complex saccharides of the *E. coli* strain used in this study.

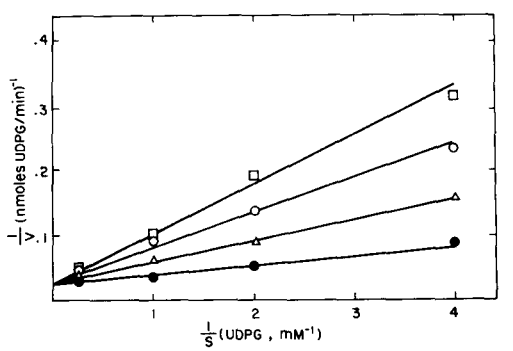


Fig. 7. Inhibition of UDPG dehydrogenase by UDPXyl at various concentrations of UDPG. Standard assay conditions were used except that the UDPG concentration was varied as shown and UDPXyl was included in the standard assay medium at the following concentrations (μM): 0, ●; 35, △; 70, ○; 100, □. 60 μg of protein (enzyme spec. act. 1.2) was used for each assay, which was run in duplicate.

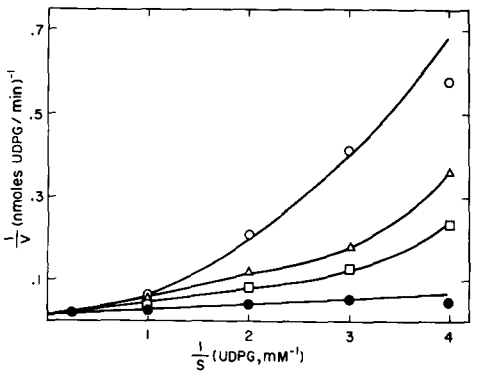


Fig. 8. Inhibition of UDPG dehydrogenase by UDP glucuronate at various concentrations of UDPG. The standard conditions of assay were used except the UDPG was varied as indicated and UDPglucuronate was included in the standard assay medium at the following concentrations (μM): o, ●; 10, □; 16.5, △; 23.3, ○. 60 μg of protein (enzyme spec. act. 1.2) was used for each assay, which was run in duplicate.

Inhibition by UDPglucuronate, on the other hand, displays cooperativity. As can be noted in Fig. 8 plots of $1/v$ *versus* $1/[UDPG]$ deflect upward at low UDPG concentrations in the presence of UDPglucuronate. Although a plot of UDPglucuronate concentration *versus* percent inhibition does not generate curves that are clearly sigmoidal, a plot of log $(V-v)/v$ *versus* log UDPglucuronate (Hill plot [17]) yields lines with average slopes (n) of 1.2. This suggests that there is some degree of cooperativity between UDPglucuronate binding sites. However, at high UDPG concentrations UDPglucuronate acts as an inhibitor competitive with UDPG (Fig. 8). This cooperative inhibitory effect of UDPglucuronate may be important in maintaining relative concentrations of UDPG and UDPglucuronate which are optimal for polysaccharide synthesis. It is of interest that with those UDPG dehydrogenases for which UDPXyl acts as a cooperative inhibitor[2–4,15,16] UDPglucuronate has no cooperative effect.

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