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URIDINE DIPHOSPHATE GLUCOSE DEHYDROGENASE OF RAT TISSUE

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SUMMARY

1. UDP-glucose dehydrogenase (UDP-glucose:NAD^+ oxidoreductase, EC 1.1.1.22) from rat liver was purified over 200-fold, and some of its properties were elucidated.

2. The enzyme has a pH optimum of 8.9; the K_m is 0.023 mM for UDP-glucose and 0.081 mM for NAD^+.

3. The reaction product, UDP-glucuronic acid, is a relatively weak competitor of UDP-glucose. Inhibition by UDP-xylose is much more pronounced.

4. Experiments with radioactive UDP-glucuronic acid demonstrate that the reaction cannot be reversed at pH 8.9 or 7.0.

5. The enzyme was also purified from rat skin.

INTRODUCTION

In order to investigate the metabolism and possible control mechanism in the synthesis of mucopolysaccharides, studies were made to characterize some of the enzymes assumed to be in the biosynthetic pathway. Previous reports dealt with the nucleoside diphosphate glucose pyrophosphorylases[1] and L-glutamine:D-fructose 6-phosphate amidotransferase (EC 2.6.1.16)[2]. The present paper describes studies on uridine diphosphate glucose dehydrogenase (UDP-glucose:NAD^+ oxidoreductase, EC 1.1.1.22) which catalyzes the reaction:

$$\text{UDP-glucose} + 2NAD^+ \rightarrow \text{UDP-glucuronic acid} + 2NADH + 2H^+$$

This enzyme, originally purified from calf liver[3], was subsequently found in a number of diverse cell types, *e.g.* plant[4], microorganisms[5,6], and rabbit skin[7]. More recently, the isolation of a homogeneous enzyme protein preparation from beef liver has been reported[8].

UDP-glucose dehydrogenase is of considerable interest because of its role in providing the uronic acid component for the synthesis of mucolopysaccharides, in

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addition to yielding glucuronic acid for conjugation reactions. Since UDP-glucose is also the substrate for the synthesis of glycogen, the dehydrogenase reaction is a branch from the principal pathway of glucose storage and utilization. It is, therefore, reasonable to expect that this reaction would be subject to various control factors. Previous studies on the mammalian enzyme dealt with preparations from beef, however, this cannot be utilized for metabolic studies in intact animals. This report describes the purification and characterization of the enzyme from rat liver. The dehydrogenase from skin is also described.

EXPERIMENTAL PROCEDURES

Analytical methods

Enzyme activity was assayed spectrophotometrically by a modification of the procedure of STROMINGER *et al.*[3]. Protein was determined according to LOWRY *et al.*[9] and by the method of WARBURG AND CHRISTIAN[10]. Uronic acid was measured by the carbazole method[11].

Chromatography and electrophoresis

The solvents for paper chromatography were (A) 0.1 M ammonium acetate, pH 7.5–ethanol (2:5, v/v); (B) 0.1 M ammonium acetate, pH 3.8–ethanol (2:5, v/v). Electrophoresis was performed with cellulose acetate strips (Gelman Co., Sepraphore II, 2.5 cm × 17.0 cm) in 0.05 M citrate buffer, pH 4.8 for 2–4 h at 300 V. Each strip was then cut in half along its axis; one half was stained for protein and the other for dehydrogenase activity. Proteins were visualized by staining with a 0.2% (w/v) solution of Ponseau-S and in 5% acetic acid. The location of enzyme was determined by incubating the strips in the dark for 30 min, at 37°, in a solution composed of the following: 0.2 mM UDP-glucose, 1.0 mM NAD^+, 0.08 M glycine buffer, pH 8.9, 0.02% (w/v) phenazine methosulfate and 0.01% (w/v) nitroblue tetrazolium. The strips were then washed with 40:1 (v/v) formaldehyde solution. Enzyme activity was visualized by the blue-purple color.

Electrophoresis on polyacrylamide gels was carried out at pH 9.5 by the method of ORNSTEIN[12] and DAVIES[13].

Electrofocusing

An enzyme sample, dialyzed overnight against 1% glycine, was applied to an LKB column (110 ml) with 1 g of ampholyte pH range 3–10 or 5–8. The current was adjusted to maintain a constant output of 0.5 W. The run was terminated when the current remained constant for several hours. The ampholyte was collected in 3 ml fractions, and the pH and absorbance at 280 nm of each fraction were determined.

Purification of liver UDP-glucose dehydrogenase

The first five steps in the purification (Table I) were similar to those employed for the beef liver enzyme[3].

Carboxymethyl cellulose. The enzyme preparation obtained after fractionation with acetone was dissolved in a pH 6 buffer composed of 0.29 mM KH_2PO_4 and 1.05 mM citric acid, and applied to a column (2 cm × 16 cm) of CM-cellulose. The column was washed with the buffer until all unadsorbed protein was eluted. A constant increase in ionic strength of eluting buffer was effected by running 0.29 M K_2HPO_4–

0.105 M citrate, pH 6, into 500 ml of the starting buffer. The enzyme fractions, which were eluted after removal of inactive protein, were combined and precipitated from 55% $(NH_4)_2SO_4$.

Sephadex G-200. A solution of the above precipitate in the dilute citrate-phosphate buffer was applied to a column (2.5 cm × 82 cm) of Sephadex G-200 and eluted with the same buffer. This resulted in the appearance of inactive protein followed by enzyme fractions. The fractions containing enzyme with a specific activity of 1000 units per ml or greater, were combined.

Hydroxylapatite fractionation. A portion of the above enzyme solution was dialyzed overnight against 5 mM phosphate buffer, pH 6.3, containing 20% glycerol and applied to a column (2 cm × 4 cm) of hydroxylapatite. The column was eluted by a stepwise gradient of phosphate buffer, pH 6.3, containing 20% glycerol, having the following molarities: 0.005, 0.05, 0.20, 1.0. The active fractions appeared on elution with 0.20 M buffer. These were combined and the enzyme was precipitated by making the solution 60% saturated with $(NH_4)_2SO_4$.

UDP-glucose dehydrogenase from skin

Rat skin was cut into small pieces, placed in liquid nitrogen and ground in a pre-cooled Wiley mill. The resultant powder was extracted three times with acetone (−20°) and air dried for 30 min. 2-g batches of this acetone powder were homogenized with 30 ml 0.4% Triton X-100 (pH 5.7) at 0–5° for 2 min and the mixture was stirred slowly for 30 min. The extracts obtained after centrifugation were stirred with 20% protamine sulfate (15 ml per 160 ml) and the resultant precipitate was discarded. The enzyme was fractionated from this solution with $(NH_4)_2SO_4$ (similar to the method for the liver enzyme) and then purified by the hydroxylapatite procedure. The enzyme fraction from this tissue was also eluted from the column with 0.20 M phosphate, pH 6.3.

RESULTS AND DISCUSSION

Purification and properties of the liver enzyme

The results of a typical isolation are shown in Table I. Electrophoresis of the product on polyacrylamide gel (pH 6.9) and on cellulose acetate (pH 4.8) showed the presence of a major protein band and a comparatively smaller additional band. In the latter system, only the major band, having the higher mobility, exhibited UDP-glucose dehydrogenase activity.

The pH optimum for the enzyme is 8.9. The isoelectric point is approximately 6.3 as determined by isoelectric focusing. KCl, LiCl, NaCl, $MgCl_2$ (10 mM); EDTA (50 mM); or NaF (0.1 mM) had no significant effect on the activity. The enzyme is strongly inhibited by 1.1 mM iodoacetate and 0.01 mM *p*-chloromercuribenzoate. Neither of the substrates were able to relieve the inhibition, suggesting that sulfhydryl groups may not be involved only in the binding site.

When UDP-glucose was replaced by 0.2–0.4 mM UDP-galactose, UDP-*N*-acetylglucosamine, GDP-glucose, ADP-glucose or glucose 1-phosphate, there was no activity. TDP-glucose showed about 3% the activity of UDP-glucose. With UDP-glucose, if the β-NAD^+ was substituted by 1.0–2.0 mM α-NAD^+ or β-$NADP^+$ there was no reaction. Nicotinamide hypoxanthine dinucleotide gave 30% of the reaction

TABLE I

PURIFICATION OF UDP-GLUCOSE DEHYDROGENASE FROM LIVER

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Activity* (units/ml)*	*Specific activity*	*Purification (-fold)*	*Recovery (%)*
1. Crude extract	7000	10.0**	200	20	—	—
2. $(NH_4)_2SO_4$	750	22.2**	2100	94.6	4.7	100
3. After acid, 50°	690	10.0**	1760	176	8.8	77
4. After alkaline $(NH_4)_2SO_4$	340	14.7***	3400	231	11.5	73
5. After acetone fractionation	122	7.5***	5600	751	37.5	48
6. CM-cellulose	24	27.0***	25000	926	46.3	44
7. Sephadex G-200	42	2.9***	6500	2240	112	27****
8. Hydroxylapatite	2	7.1***	34000	4788	239	20****

* The units are those defined by STROMINGER, *et al.*[3].
** Protein determined by LOWRY *et al.* procedure.
*** Protein determined by spectrophotometric method.
**** Calculated value, since only a fraction from the previous step was employed.

of β-NAD^+. The enzyme is thus specific for the glycosyl and nucleoside moieties of the nucleotide sugar but has a lesser specificity with respect to the purine moiety.

Identification of reaction product

UDP-glucuronic acid was identified after incubation of a reaction mixture, by chromatography and by colorimetric identification[11]. For the former, a mixture composed of 0.60 ml 1 mM UDP-glucose, 0.60 ml 5 mM NAD^+, 0.60 ml 0.5 M glycine buffer, pH 8.9, and 0.03 ml enzyme protein (54 units) was incubated for 2 h. After heat denaturation, the supernatant fluid was concentrated to 0.1 ml and aliquots along with appropriate controls and standards were chromatographed in Solvent systems A and B. Ultraviolet-absorbing material having a mobility identical with UDP-glucuronic acid was observed only in the incubated reaction mixture and was absent in the zero-time control. Colorimetric determination for uronic acid of similar mixtures gave positive results only in the incubated solutions.

Reversibility

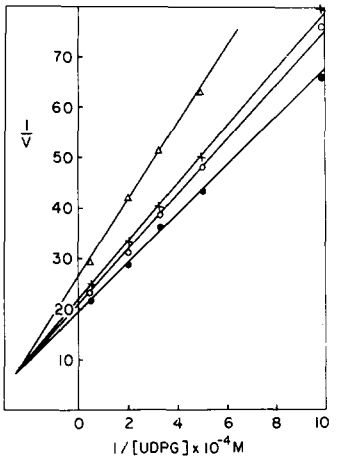
No oxidation of NADH could be detected spectrophotometrically when it was incubated with enzyme and UDP-glucuronic acid at pH 8.5 or pH 7.

In other experiments, 0.01 μmole UDP-glucuronic acid, uniformly labeled with ^{14}C on the uronic acid, 233 mC/mmole, 0.40 μmole NADH, 15 μmoles glycine buffer and 26 units of enzyme protein in a total volume of 0.15 ml were incubated for 2 h at pH 8.5 and 7.0. Chromatography of 20-μl aliquots in Systems A and B and assay of sections of the paper for radioactivity, showed no detectable activity in the area of UDP-glucose.

Effect of substrate concentration

The K_m for UDP-glucose obtained from double reciprocal plots of initial velocity versus concentration, is 0.023 mM. The K_m for NAD^+ is 0.081 mM.

Additional detailed investigations were made on the kinetic characteristics of



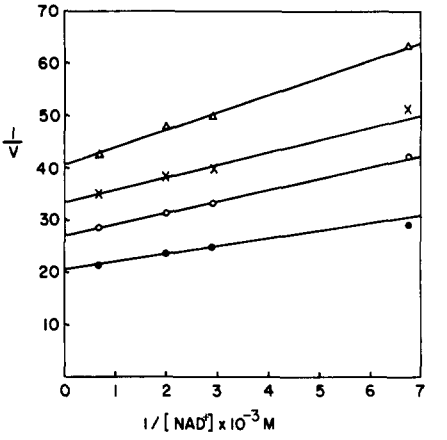


Fig. 1. Double reciprocal plots of initial velocity *versus* UDP-glucose (UDPG) concentration at various constant levels of NAD^+. The concentrations of NAD^+ were: ●, 1.48 mM; ○, 0.49 mM; ×, 0.35 mM; △, 0.15 mM.

Fig. 2. Double reciprocal plots of initial velocity *versus* concentrations of NAD^+ at various constant levels of UDP-glucose. The concentrations of UDP-glucose were: ●, 0.20 mM; ○, 0.05 mM; ×, 0.03 mM; △, 0.02 mM.

the reaction. A study on the effect of UDP-glucose concentration in the presence of a series of different fixed concentrations of NAD^+ gave the results shown in Fig. 1. This pattern is consistent with a sequential mechanism for the reaction[14]. A reciprocal

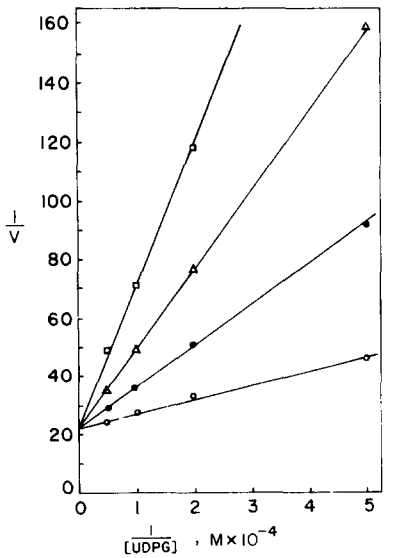


Fig. 3. Dependence of reaction rate on the concentration of UDP-glucose (UDPG) when the reaction is performed in the presence of UDP-glucuronic acid. Double reciprocal plot of initial rate *versus* concentration of UDP-glucose at saturating NAD^+ concentration. UDP-glucuronic acid concentrations: ○, none; ●, 0.3 mM; △, 0.6 mM; □, 1.2 mM.

plot of the effect of NAD^+ concentration in the presence of a series of different fixed concentrations of UDP-glucose is shown in Fig. 2.

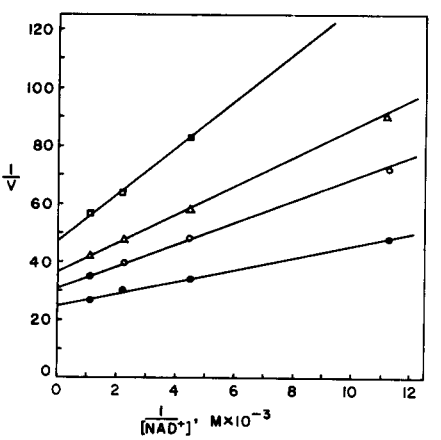
Product inhibition

Excess UDP-glucose (0.6 mM) or NAD (3.0 mM) had no inhibiting effect. However, the products of the reaction, NADH and UDP-glucuronic acid, did inhibit the reaction. As seen in Fig. 3, UDP-glucuronic acid is a relatively weak competitive inhibitor of UDP-glucose (K_i 0.14 mM). The reason is probably that the ionized carboxyl group does not allow for efficient interaction with the UDP-glucose binding site of the enzyme. Inhibition by UDP-glucuronic acid has also been reported for UDP-glucose dehydrogenase from other sources[5,15].

The inhibition of NAD^+ by UDP-glucuronic acid is shown in Fig. 4. The fact that the lines intersect at a point above the x-axis suggests that the inhibition is of a mixed type rather than strictly non-competitive. Conceivably, UDP-glucuronic acid, through its adenosine moiety, affects the binding site of NAD^+ as well as that of the UDP-glucose. The inhibition by NADH is competitive with NAD^+ (K_i 0.025 mM) as seen from Fig. 5.

Effect of hydroxylamine

Since the conversion of UDP-glucose to UDP-glucuronic acid involves the oxidation at C-6 from a hydroxyl to a carboxylic acid, it is reasonable to assume that there is an aldehyde intermediate in the reaction. In order to trap such an intermediate, studies were performed in the presence of NH_2OH. It was found that 10 mM NH_2OH did not affect the initial reaction rate, however, after 3 min it became progres-



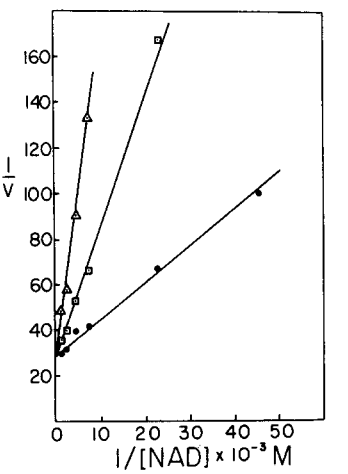
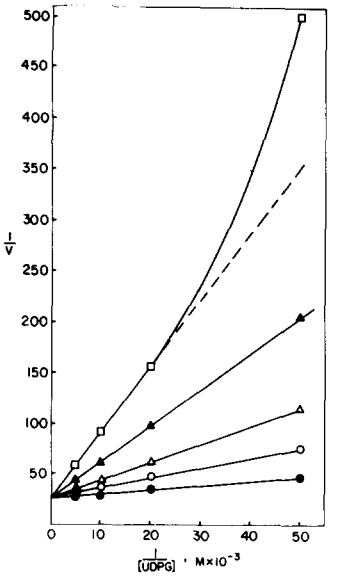


Fig. 4. Dependence of reaction rate on the concentration of NAD^+ when the reaction is performed in the presence of UDP-glucuronic acid. Double reciprocal plot of initial rate *versus* concentration of NAD^+ at saturating UDP-glucose concentration. UDP-glucuronic acid concentrations: ●, none; ○, 0.3 mM; △, 0.4 mM; □, 1.2 mM.

Fig. 5. Inhibition of UDP-glucose dehydrogenase activity by NADH. Double reciprocal plot of initial velocity *versus* concentration of NAD^+ by the following concentrations of NADH: ●, none; □, .03 mM; △, 0.10 mM.



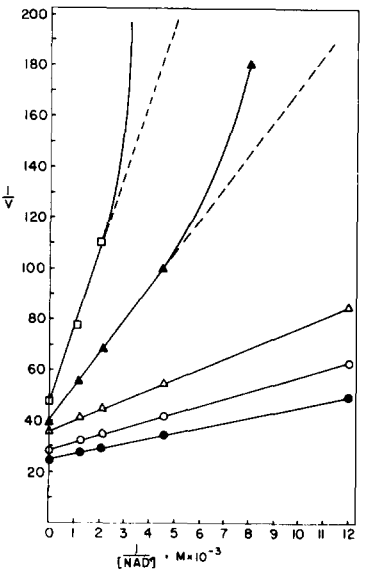


Fig. 6. Inhibition by UDP-xylose. Double reciprocal plot showing its effect on the dependence of initial velocity on UDP-glucose (UDPG) concentration. The molarities of UDP-xylose were: ●, none; ○, $1 \cdot 10^{-5}$; △, $2 \cdot 10^{-5}$; ▲, $4 \cdot 10^{-5}$; □, $6 \cdot 10^{-5}$. The broken line indicates the relationship expected in the absence of an allosteric effect.

Fig. 7. Double reciprocal plot showing the effect of UDP-xylose on the dependence of rate on NAD^+ concentration. The molarities of UDP-xylose were: ●, none; ○, $1 \cdot 10^{-5}$; △, $2 \cdot 10^{-5}$; ▲, $4 \cdot 10^{-5}$; □, $6 \cdot 10^{-5}$. With the latter, the observed value of $1/v$ (not shown in the diagram) was 300 for $1/[NAD^+]$ of $4.8 \cdot 10^3$. The broken line indicates the relationship expected in the absence of an allosteric effect.

sively less than the control. Preincubation of the enzyme with NH_2OH resulted in its deactivation. This reagent, therefore, cannot be employed simply to remove an intermediate. The action of NH_2OH in interacting with proteins and cleaving certain linkages has been reported[16,17].

Inhibition by UDP-xylose

UDP-xylose is a relatively strong competitive inhibitor (K_i 8.3 μM) of the action of the enzyme on UDP-glucose (Fig. 6). At higher concentrations of inhibitor, there are significant deviations from linearity in the reciprocal plot. The effect of UDP-xylose on initial velocity as the concentration of NAD^+ is increased is shown in Fig. 7. At lower concentrations of UDP-xylose, the inhibition is non-competitive. Higher concentrations of inhibitor produce significant deviations from linearity.

Similar inhibition by UDP-xylose has been reported for UDP-glucose dehydrogenase from several other sources[5,15]. However, with the rat liver enzyme deviation from linearity occurred only when considerably higher concentrations of UDP-xylose were employed. The possibility that inhibition by UDP-xylose may be a regulatory feed-back system has been proposed[15] and the consequences of such allosteric inhibition in the biosynthesis of the *Cryptococcus laurentii* polysaccharide has been discussed[5]. The K_i for UDP-xylose with the Cryptococcus enzyme is about half that for the

enzyme from rat liver. This, in addition to the considerable difference in the allosteric effect, suggests that regulation of the liver enzyme by UDP-xylose is confined to a minor pathway in the utilization of UDP-glucuronic acid. The primary requirements for UDP-glucuronic acid in rat liver are probably for the formation of glucuronides and ascorbic acid, rather than as a precursor for UDP-xylose. It is conceivable that in connective tissue and in skin a considerable portion of UDP-glucuronic acid is utilized for the synthesis of UDP-xylose. Regulation of the UDP-glucose dehydrogenase by this product may thus be an important factor in these tissues.

UDP-glucose dehydrogenase from skin

The original skin extract could not be assayed precisely by the standard procedure because the reaction mixture showed a considerable increase in absorbance at 340 nm even in the absence of substrate. A further complication was that the tissue extract also contained components which oxidized NADH. The minimal value for purification after the ammonium sulfate step was 4-fold, and after the hydroxylapatite procedure was 10-fold.

The pH optimum, substrate specificity and effect of iodoacetate were similar to that found for the liver enzyme. The enzyme is also inhibitable by UDP-xylose. Precise quantitative measurements on this effect await further purification of the enzyme.

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