

## Enzymatic Synthesis of Uridine 5'-Diphosphoglucuronic Acid on a Gram Scale

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A practical route to uridine 5'-diphosphoglucuronic acid (UDP-GlcUA) from uridine 5'-diphosphoglucose (UDP-Glc) on a 1-g scale has been developed using uridine 5'-diphosphoglucose dehydrogenase (UDP-Glc DH, EC 1.1.1.22) from bovine liver. Crude UDP-Glc dehydrogenase was isolated from beef liver (450 units from 2.4 kg of frozen liver). Commercially available UDP-Glc dehydrogenase as well as a preparation from calf liver acetone powder were also evaluated as catalysts for large-scale production of UDP-GlcUA: both preparations exhibited too little activity to be synthetically useful. A platinum-catalyzed oxygen oxidation of UDP-Glc was also examined as a possible route to UDP-GlcUA: enzymatic oxidation was superior. These results establish a route to another of the important activated monosaccharides required for cell-free enzymatic syntheses of mammalian oligo- and polysaccharides.

### Introduction

Oligo- and polysaccharides are important in mammalian biochemistry.<sup>1-4</sup> Originally considered primarily as structural components of cells and as energy-storage vehicles, many additional roles for carbohydrates are now established. They are participants in biological communication events, including cell-cell signaling during growth and differentiation,<sup>5,6</sup> and are cell markers in several diseases.<sup>7</sup> Cell-surface glycoconjugates are recognition sites for the binding of hormones, soluble lectins and toxins, and adhesion sites in bacterial<sup>8</sup> and viral infection.<sup>9</sup> A variety of carbohydrate polymers also display important biological activities, including immunostimulation,<sup>10</sup> inhibition of coagulation,<sup>11</sup> and protection from viral infection.<sup>12</sup>

With the recognition of the importance of carbohydrates in vivo has come an intensified demand for practical synthetic methodologies for the preparation of well-defined oligosaccharides. Although the field of glycosidic bond formation has advanced rapidly,<sup>13-17</sup> new strategies for monosaccharide coupling are of great interest.<sup>18-20</sup> En-

zymes are useful catalysts in organic synthesis<sup>21-25</sup> and are now being applied to carbohydrate synthesis.<sup>26,27</sup> In this latter capacity, enzymes possess two attractive attributes. First, enzymes operate in aqueous solution, where carbohydrates are soluble in their unprotected form; the lengthy protection/deprotection schemes that are often required in conventional carbohydrate syntheses are thus unnecessary. Enzymes are often specific. This specificity is of obvious value in manipulating the rich stereochemistry of carbohydrates. In short, enzymes are well-suited to carbohydrate synthesis.

In vivo, the glycosidic bonds of most oligosaccharides are formed enzymatically using monosaccharides activated as nucleoside phosphate sugars. In mammalian biochemistry, eight monosaccharides comprise the bulk of the sugars found in glycoconjugates. In their activated form, these sugars are as follows: uridine 5'-diphosphoglucose (UDP-Glc), uridine 5'-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), uridine 5'-diphosphogalactose (UDP-Gal), uridine 5'-diphospho-*N*-acetylgalactosamine (UDP-GalNAc), uridine 5'-diphosphoglucuronic acid (UDP-GlcUA), guanosine 5'-diphosphomannose (GDP-Man), guanosine 5'-diphosphofucose (GDP-Fuc), and cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc). The enzymes that form the activated nucleoside sugars and transfer them into nascent oligosaccharide chains are collectively referred to as the enzymes of the Leloir pathway. Leloir-pathway enzymes have been used as catalysts for glycosidic bond formation in a number of synthetic applications.<sup>26,28-30</sup>

In order for enzyme-based synthetic methodologies to be feasible, a source of activated monosaccharides is required. Although all eight nucleoside phosphate sugars are available commercially, all are too expensive for synthetic use. We have undertaken a program to develop practical gram-scale syntheses of the eight activated sugars

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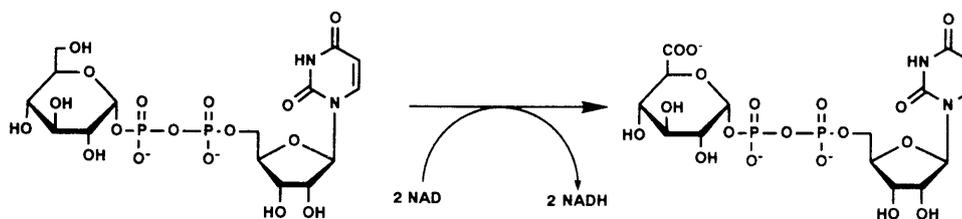
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**Scheme I. Uridine 5'-Diphosphoglucose Dehydrogenase (UDP-Glc DH) Catalyzed Conversion of UDP-Glc to UDP-GlcUA in the Presence of NAD<sup>+</sup>**



utilized in mammalian biochemistry. To date, we have reported multi-gram syntheses of UDP-Glc,<sup>31</sup> UDP-Gal,<sup>31</sup> and CMP-NeuAc<sup>32</sup> and convenient syntheses of their precursors, the nucleoside triphosphates.<sup>33</sup> We now report a convenient, gram-scale synthesis of UDP-GlcUA using UDP-Glc dehydrogenase isolated from frozen bovine liver. UDP-GlcUA has previously been prepared on a small scale by enzymatic oxidation using crude enzyme preparations from *Bacillus*.<sup>34</sup>

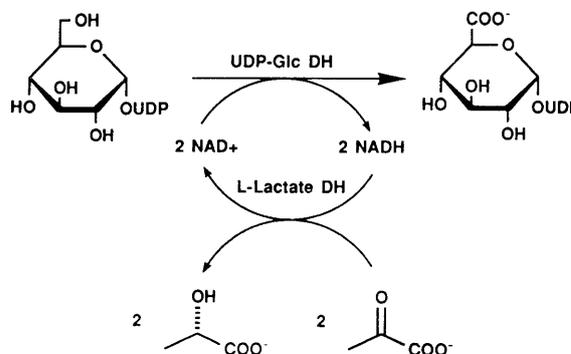
Glucuronic acid is found *in vivo* primarily as a solubilizing agent for insoluble xenobiotics and as a constituent of the glycosaminoglycan family of polysaccharides (mucopolysaccharides). The glycosaminoglycans, linear repeating polymers of *N*-acetylglucosamine and uronic acids, include such important compounds as hyaluronic acid, chondroitin and chondroitin sulfate, and heparin. A cell-free enzymatic approach to the synthesis of these compounds is attractive both from the point of view of synthesizing quantities of them and for the preparation of new materials. We have previously used synthetic UDP-GlcUA in a cell-free synthesis of hyaluronic acid.<sup>35</sup>

## Results and Discussion

**Enzymatic Oxidation of UDP-Glc.** Our initial approach to UDP-GlcUA involved the use of UDP-Glc dehydrogenase from bovine liver (EC 1.1.1.22). This nicotinamide-dependent enzyme catalyzes the four-electron oxidation of UDP-Glc to UDP-GlcUA (Scheme I).

UDP-Glc dehydrogenase is commercially available, although it is expensive (\$20/U, Sigma: a unit of enzyme is the amount required to catalyze the conversion of 1  $\mu$ mol of reactants to products per minute under optimal conditions of temperature and pH). We investigated the utility of the commercial enzyme for large-scale production of UDP-GlcUA. Reactions were carried out in distilled water, 0.1 M Tris, or 0.1 M glycine at pH 8.7, the reported optimal pH for UDP-Glc DH.<sup>36</sup> Oxidations were typically carried out on a 0.15 mmol scale using 2 U of enzyme. The reaction can be conveniently followed by ion-exchange HPLC with UV detection. Under these conditions, approximately 10% conversion of UDP-Glc to UDP-GlcUA was observed after 2 days, with a limiting value of 30% conversion being reached after 7 days. Although both UDP-GlcUA and NADH are reported to be competitive inhibitors of UDP-Glc dehydrogenase, with  $K_i$  values of

**Scheme II. Gram-Scale Production of UDP-GlcUA (Oxidized NAD<sup>+</sup> Regenerated Using a Coupled Lactate Dehydrogenase Reduction. A 2-Fold Excess of Pyruvate Was Used To Ensure Complete Reaction)**



50 and 6  $\mu$ mol, respectively,<sup>37</sup> the primary problem was apparently enzyme degradation: while the addition of UDP-Glc failed to stimulate reaction, the addition of enzyme resulted in the appearance of additional product. In light of the significant cost of the enzyme, this procedure was unsuitable for gram-scale production of UDP-GlcUA.

We therefore investigated methods to isolate the enzyme in a more useful form. That is, one that would give either more stable enzyme or a larger quantity of enzyme. We investigated a literature preparation of UDP-Glc dehydrogenase from calf-liver acetone powder.<sup>38</sup> Although this preparation does not yield significantly more enzyme than is feasible to use from commercial sources, we hoped that the isolated UDP-Glc dehydrogenase might be more suitable for synthetic use. By use of the literature preparation, 10 U of enzyme was isolated from 20 g of commercial calf-liver acetone powder. The isolated enzyme behaved similarly to the commercial enzyme, and this route was not pursued further.

A convenient preparation of UDP-Glc dehydrogenase from whole, fresh bovine liver has also been reported.<sup>37,39</sup> Frozen bovine liver was thawed, cut into 4-cm-square pieces, and homogenized. Connective tissue and fat were removed by centrifugation and filtration through cotton. The filtered supernatant was precipitated with solid ammonium sulfate. The pellet was redissolved in buffer, acidified to pH 4.9, and heated to 60 °C for 1 min. The precipitate was discarded, and the supernatant was reprecipitated with alkaline ammonium sulfate. The preparation yielded 450 U of enzyme from 2.4 kg of frozen liver.<sup>40</sup> The specific activity of this preparation was approximately 0.04 U/mg protein. Although 450 U is

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somewhat lower than the literature value,<sup>39</sup> the use of fresh liver would almost certainly improve the yield.

UDP-Glc dehydrogenase isolated in this way was used to prepare UDP-GlcUA from UDP-Glc on a 1-g scale. UDP-Glc was dissolved in 50 mM Tris buffer, pH 7.5, with 100 U of crude UDP-Glc dehydrogenase. A catalytic amount of NAD was added to the reaction; oxidized cofactor was regenerated using a coupled pyruvate/lactate dehydrogenase system (Scheme II). To ensure complete conversion of UDP-Glc to UDP-GlcUA, 4 equiv of pyruvate were employed. UDP-GlcUA was purified from the reaction mixture by gel filtration chromatography on Sephadex G25: the product eluted after approximately 1.8–1.9 bed volumes. The pooled, product-containing fractions were lyophilized to yield a pale yellow powder. The material was indistinguishable from commercial UDP-GlcUA by <sup>1</sup>H and <sup>13</sup>C NMR. Acetic acid from the UDP-Glc dehydrogenase buffer was the only major contaminant and could be removed by ion-exchange chromatography. The mass recovery was 87%, based on UDP-Glc. Synthetic UDP-GlcUA was assayed enzymatically using commercially available UDP-glucuronyltransferase (EC 2.4.1.17). This assay gave a purity of 84%. Since no observable impurities appeared in the <sup>1</sup>H NMR spectrum, the primary impurity is most probably water.

**Platinum-Catalyzed Air Oxidation.** Glucose can be selectively oxidized at C6 by a platinum-catalyzed oxygen oxidation: this oxidation is important in commercial syntheses of ascorbic acid.<sup>41</sup> More recently, a report appeared on the successful oxidation of glucose 1-phosphate to glucuronic acid 1-phosphate using a similar approach.<sup>42,43</sup> We therefore examined the feasibility of preparing UDP-GlcUA from UDP-Glc via a platinum-catalyzed oxidation on a small (0.15 mmol) scale. A brief survey confirmed that oxidation of UDP-Glc is effective only above pH 9.0. Although initially a high rate of conversion of UDP-Glc to UDP-GlcUA was observed, rapid catalyst inactivation occurred, as well as decomposition of UDP-Glc. A variety of catalyst systems were examined: in all cases decomposition at elevated pH was the predominant reaction. Although the products of decomposition were not unambiguously identified, base-catalyzed decomposition of UDP-Glc has been reported previously.<sup>44</sup>

These results suggest that a platinum-catalyzed air oxidation is not feasible for the production of gram quantities of UDP-GlcUA. Although it may be possible to prevent deactivation of the platinum by careful preparation of the catalyst,<sup>42,43</sup> the oxidation would still have to be carried out at elevated pH, where UDP-Glc is unstable.

### Conclusions

We have developed a method for the production of gram quantities of UDP-GlcUA from UDP-Glc. The method involves a straightforward enzyme isolation from a convenient source. Although the cost of commercial UDP-Glc is significant (\$70/g), UDP-Glc can be prepared on a large scale from inexpensive glucose-6-phosphate by literature methods.<sup>31</sup>

This enzymatic method is superior to other methodologies. Although platinum-catalyzed oxidations could be improved by appropriate catalyst preparation, difficulties associated with catalyst preparation and base-catalyzed

decomposition of UDP-Glc would still render this route inferior to enzymatic preparation. Another potential route, chemical synthesis from glucuronic acid, would almost certainly be more expensive and less efficient than enzymatic synthesis.<sup>45</sup>

### Experimental Section

All chemicals were reagent grade and used without further purification. Enzymes were purchased from Sigma. Water was distilled from glass in a Corning AG-1b still. HPLC analyses were performed using a Waters 600E solvent delivery system.

**Isolation of UDP-Glc Dehydrogenase from Frozen Bovine Liver.** UDP-Glc dehydrogenase was purified according to the method of Zalitis and Feingold.<sup>36,38</sup> "Buffer" refers to 0.01 M NaOAc, 0.002 M EDTA, 0.01 M 2-mercaptoethanol, pH 5.5 throughout. Protein concentrations were determined by the method of Lowry.<sup>46</sup>

Frozen bovine liver was obtained from PelFreeze Co., AR, and thawed wrapped in plastic in 4 °C water. The liver was cut into 4-cm-square pieces. Liver (300-g lots, total of 2400 g) was homogenized with buffer (300 mL per lot of liver) in a Waring blender. The resulting suspension was centrifuged (10000g, 30 min), and the supernatant was filtered through cotton. Solid ammonium sulfate (517 g, 30% saturation) was added to the supernatant (total of 3100 mL). The suspension was centrifuged (10000g, 30 min) and the pellet discarded. Additional ammonium sulfate (375 g, to 50% saturation) was added. The suspension was centrifuged (10000g, 30 min), and the pellet was redissolved in buffer (600 mL) to give a protein concentration of approximately 120 mg/mL. The solution was divided into 200-mL lots, the pH was adjusted to 4.9 with 1 M acetic acid, and the solution was heated to 60 °C in a water bath. The temperature was maintained at 60 °C for 2 min then lowered to 2 °C in a salt-ice bath. The suspension was centrifuged (10000g, 30 min), and the pellet was discarded. Solid ammonium sulfate (125 g, 35% saturation) was added, and the suspension was centrifuged (10000g, 30 min). Additional ammonium sulfate (38 g, to 45% saturation) was added. The suspension was centrifuged (10000g, 30 min), and the pellet was redissolved in buffer (200 mL) to give a protein concentration of approximately 55 mg/mL. The enzyme was used without further purification.

**Preparation of UDP-GlcUA Using UDP-Glc Dehydrogenase from Bovine Liver.** UDP-Glc (Sigma, 1 g, 1.5 mmol) was dissolved in Tris buffer (50 mM, pH 8.7, 30 mL). To this was added NAD (50 mg, 0.08 mmol), pyruvate (0.53 g, 6 mmol), and lactate dehydrogenase (EC 1.1.1.27, Sigma, from rabbit muscle, 200 units). The pH was readjusted to 8.7, and UDP-Glc dehydrogenase (150 U) was added. The reaction was monitored by HPLC (Partisil 10 SAX strongly basic anion exchange column, 1.6 mL/min, linear gradient of 0.007 M phosphate, pH 4.7 to 1.1 M KCl in 0.007 M phosphate, pH 4.7 during 30 min, UV detection at 264 nm). Typical elution times under these conditions were 8.2 and 14.3 min for UDP-Glc and UDP-GlcUA, respectively. After 12 h, no UDP-Glc was visible; UDP-GlcUA was the sole major product. The reaction mixture was concentrated to 10 mL by rotary evaporation, applied to a column of Sephadex G25, and eluted with distilled water. Fractions were detected by UV at 264 nm. Fractions containing UDP-GlcUA were combined and lyophilized to give a slightly yellow powder (890 mg, 87%). <sup>1</sup>H and <sup>13</sup>C NMR spectra were indistinguishable from authentic material (Sigma): <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz, HOD at δ 4.63)<sup>47</sup> δ 7.815 (d, *J* = 8.12 Hz, 1 H), 5.852 (m, 2 H), 5.489 (dd, *J*<sub>1</sub> = 3.47 Hz, *J*<sub>2</sub> = 4.13 Hz, 1 H), 4.238 (m, 2 H), 4.152 (m, 1 H), 4.114–4.031 (m, 1 H), 3.998 (m, 2 H), 3.65 (t, *J* = 9.48 Hz, 1 H), 3.451 (dt, *J*<sub>1</sub> = 9.83 Hz, *J*<sub>2</sub> = 3.12 Hz, 1 H), 3.38 (t, *J* = 9.67 Hz, 1 H); <sup>13</sup>C NMR (D<sub>2</sub>O, 125 MHz, dioxane at δ 67.3) δ 177.1, 166.9, 152.5, 142.3, 103.4, 96.1, 89.0, 83.9, 74.5, 73.6, 73.3, 72.5, 72.0, 70.4, 65.5.

**Enzymatic Assay of UDP-GlcUA.** Enzymatic assay of UDP-GlcUA is carried out by observing the transfer of GlcUA

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from UDP-GlcUA to phenolphthalein. The GlcUA-phenolphthalein conjugate does not absorb at 540 nm. The assay was a modified version of a procedure obtained from Sigma Chemical Co.

A stock solution of Tris (0.3 M), phenolphthalein (1 mM), MgCl (45 mM), bovine serum albumin (3 mg/mL), and  $\beta$ -mercaptoethanol (3 mM) was prepared in distilled water, and the pH was adjusted to 8.0. A solution of  $\sim 0.2$  U/mL uridine 5'-diphosphoglucuronyltransferase (EC 2.4.1.17, from bovine liver, Sigma) was prepared in distilled water. A 0.3 mM solution of UDP-GlcUA was also prepared. Two solutions were prepared as follows:

	solution 1	solution 2
stock solution	6.3 mL	6.3 mL
enzyme	0.2 mL	0.0 mL
distilled water	0.0 mL	0.2 mL
UDP-GlcUA	0.5 mL	0.5 mL

Enzymatic transfer of GlcUA from UDP-GlcUA to phenolphthalein was initiated by addition of the enzyme. Aliquots of 0.25 mL were withdrawn at intervals and quenched into 1.5 mL of 95% ethanol. The solutions were centrifuged (13000g, 5 min), 0.5 mL of supernatant was added to 4 mL of glycine buffer (0.2 M, pH 10.4), and the absorbance at 540 nm was recorded. The reaction was allowed to proceed until no further change in  $A_{540}$  was observed. The concentration of UDP-GlcUA was calculated from the difference between the final  $A_{540}$  from solution 1 and the  $A_{540}$  from solution 2, utilizing a molar absorptivity for phenolphthalein at pH 10.4 of  $38\,500\text{ L mol}^{-1}\text{ cm}^{-1}$ .

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**Registry No.** UDP-Glc, 133-89-1; UDP-GlcUA, 2616-64-0; UDP-Glc DH, 9028-26-6; UDP-glucuronyltransferase, 9030-08-4; pyruvate DH, 9014-20-4; lactate DH, 9001-60-9.