The pyrophosphorylase that condenses UTP and GlcNAc-1-P was purified 9500-fold to near homogeneity from the soluble fraction of pig liver extracts. At the final stage of purification, the enzyme was quite stable and could be kept for at least 4 months in the freezer with only slight loss of activity. On native gels, the purified enzyme showed a single protein band, and this band was estimated to have a molecular mass of about 64 kDa on Sephacryl S-300. SDS-polyacrylamide gel electrophoresis of the enzyme gave three protein bands of 64, 57, and 49 kDa, but these polypeptides are all closely related based on the following. 1) All three polypeptides show strong cross-reactivity with antibody prepared against the 64-kDa band. 2) All three proteins become labeled with either the UDP-GlcNAc photoaffinity probe azido-125I-salicylate-allylamine-UDP-GlcNAc or a similar UDP-GalNAc photoaffinity probe, and either labeling was inhibited in a specific and concentration-dependent manner by unlabeled UDP-GlcNAc or UDP-GalNAc. Thus, the enzyme is probably a homodimer composed of two 64-kDa subunits. The purified enzyme had an unusual specificity in that, at higher substrate concentrations, it utilized UDP-GalNAc as a substrate as well as UDP-GlcNAc in the reverse direction and GalNAc-1-P as well as UDP-GlcNAc in the reverse direction. However, the $K_m$ for the GalNAc substrates was considerably higher than that for GlcNAc derivatives. This activity for synthesizing UDP-GalNAc was not due to epimerase activity since no UDP-GalNAc could be detected when the enzyme was incubated with UDP-GlcNAc for various periods of time. The pyrophosphorylase required a divalent cation, with Mn$^{2+}$ being best at 0.5-1 mM, and the pH optimum was between 8.5 and 8.9.

N-Acetylglucosamine (GlcNAc) is a very important sugar in complex carbohydrates since it is a component of N-linked oligosaccharides (1), O-linked oligosaccharides (2), and glycolipids (3) and recently has been demonstrated to be linked in O-glycosidic linkage to serine and threonine residues on nuclear proteins (4) and perhaps some other proteins. Thus, a key enzyme in the production of GlcNAc polymers is UDP-GlcNAc pyrophosphorylase, the enzyme that catalyzes the formation of UDP-GlcNAc via the following reaction: $\text{UTP} + \text{GlcNAc-1-P} \rightarrow \text{UDP-GlcNAc} + \text{PP}_i$.

UDP-GlcNAc pyrophosphorylase was first partially purified from calf liver and from Staphylococcus aureus by Strominger and Smith (5), and various properties of the enzyme were determined. Those enzyme preparations utilized UDP-GalNAc as a substrate at about 2-8% of the rate of the pyrophosphorylase and UDP-GlcNAc, but it was not clear whether that activity was due to a contaminating pyrophosphorylase. With the partially purified enzyme, the rate of UDP-glucose pyrophosphorylation was about 30% of the rate with UDP-GlcNAc (5). The pyrophosphorylase was also partially purified from calf brain and utilized UDP-glucose at about 36% of the rate with UDP-GlcNAc (6). However, there have been no reports on the purification of UDP-GlcNAc pyrophosphorylase to homogeneity. This report describes the purification of the pig liver enzyme to homogeneity and the isolation of three peptides of 64, 57, and 49 kDa by SDS-PAGE.

Based on cross-reactivity with antibody prepared against the 64-kDa protein as well as labeling studies with two different photoaffinity analogs, these smaller peptides are probably proteolytic degradation products of the 64-kDa band. However, these smaller peptides are apparently produced even in the presence of a mixture of protease inhibitors, and their presence in the enzyme preparation may contribute to the high $K_m$ values observed with some of the substrates. The native enzyme is probably a homodimer of two 64-kDa polypeptides. This enzyme appears to be quite distinct from other reported UDP-GlcNAc pyrophosphorylases in terms of pH optima, substrate specificity, and other properties, including its reactivity with GalNAc-1-P and UDP-GalNAc. The enzyme should prove useful in the preparation of radiolabeled UDP-hexosamines and in the preparation of photoaffinity analogs for the study of GlcNAc- and GalNAc-transferases.

### EXPERIMENTAL PROCEDURES

### Materials

UDP-[3H]GlcNAc (6 Ci/mmol) and [5,6-3H]UTP (30 Ci/mmol) were purchased from ICN. All other nucleoside mono-, di-, and triphosphates as well as sugar phosphates were obtained from Sigma. The following enzymes were also from Sigma: glucose-6-phosphate dehydrogenase (EC 1.1.1.49), nucleoside-diphosphate kinase (EC 2.7.4.6), hexokinase (EC 2.7.1.1), inorganic pyrophosphatase (EC 3.6.1.1), and alkaline phosphatase (EC 3.1.3.1). Various adsorbents were obtained from the following sources: DEAE-cellulose (DE52) from Whatman; Sephadex G-200, Polybuffer Exchanger 94, and heparin-Sepharose from Pharmacia Biotech Inc.; and Sephacryl S-300, blue Sepharose, phenyl-Sepharose, UTP-Sepharose, UDP-hexanolamine-Sepharose, IDA-Sepharose, and protein A-Sepharose from Sigma. Polyethyleneimine-cellulose TLC plates were purchased from EM Science, cellulose TLC plates were from Eastman Kodak Co., and silica gel TLC plates were from Merck.

1 The abbreviations used are PAGE, polyacrylamide gel electrophoresis; IDA, iminodiacetic acid; MES, 4-morpholineethanesulfonic acid; 125I-ASA, azido-125I-salicylate-allylamine.
following materials were obtained from Calbiochem that included the following: 4-(2-aminoethyl)benzenesulfonfonyl fluoride, aproatin, benzamidine, EDTA, leupeptin, and procaine, a mixture of protease inhibitors obtained from reliable commercial sources and were of the best grade available.

Pyrophosphorylase Assay

UDP-HexNac pyrophosphorylase could be assayed either in the forward direction (i.e. synthesis of UDP-GlcNac (GalNac)) or in the reverse direction of UTP formation (UTP). When measured in the reverse direction, either of the following assays could be used.

Direct Assay—In this case, pyrophosphorylase activity was measured by determining the production of [3H]GlcNAc-1-P from UDP-[3H]-GlcNac and inorganic pyrophosphate essentially as described previously (5). The incubation mixtures contained the following components in a final volume of 50 μl: 5 mM sodium pyrophosphate, 2 mM UDP-[3H]-GlcNac (GalNac) (100,000 cpm), 100 mM Tris-HCl, pH 8.5, 2 mM MgCl₂, and various amounts of enzyme at the different stages of purification. After a 5-min incubation at 37°C, the reactions were terminated by the addition of 0.5 ml of 5% trichloroacetic acid, and the nucleotides were separated on DEAE-cellulose (3.5 × 35 cm) was washed with 1× Tris-HCl, pH 7.5, until the pH was 7.5, followed by equilibration with Buffer B. One-half of the dialyzed ammonium sulfate fraction was added to each column. The columns were washed with Buffer B until the effluent was clear and colorless, and the pyrophosphorylase was eluted with a 1 liter of a 0–200 mM gradient of NaCl. Fractions were assayed for enzyme activity, and active fractions were pooled and concentrated to −35 ml on an Amicon apparatus with a PM membrane.

Step 4: Gel Filtration Chromatography—The concentrated fraction from the DEAE-cellulose column was applied to a 6 × 95-cm column of Sephadex G-200 equilibrated with Buffer A. Nine-ml fractions were collected at a flow rate of 30 ml/h, and every other fraction was assayed for activity. Active fractions were pooled and concentrated to −12 ml on an Amicon filtration apparatus.

Step 5: Pentyl-Sepharose Chromatography—The active peak from Sephadex was brought to 10% saturation by the addition of solid ammonium sulfate and was applied on a 2.5 × 20-cm column of Pentyl-Sepharose equilibrated with 10% ammonium sulfate in Buffer A. The column was washed with the same solution, and 5-ml fractions were collected at a flow rate of 20 ml/h. Active fractions were pooled, concentrated to −5 ml, and dialyzed against 1 liter of Buffer A, with three changes of the dialysis fluid.

Step 6: Heparin-Sepharose Chromatography—The enzyme fraction from the previous step was applied to a 2.5 × 25-cm column of heparin-Sepharose equilibrated with Buffer A. The enzyme was eluted from the column with 500 ml of a 0–0.5 M gradient of NaCl. The active fractions were pooled, concentrated to −5 ml, and dialyzed for 6 h against Buffer C (50 mM MES, pH 7.4, containing 1 M NaCl).

Step 7: Immobilized Metal Affinity Chromatography—A 1.4 × 20-cm column of Ni⁺⁺-charged column, containing iminodiacetic acid covalently bound to Sepharose (IDA-Sepharose), was prepared and washed with Buffer C. The enzyme fraction from Step 6 was applied to this column, and the pyrophosphorylase activity was eluted with a 300-ml gradient of Buffer D (100 mM acetic buffer, pH 5.0, containing 1 M NaCl). Fractions of 5 ml were collected, and each fraction was analyzed for protein, enzymatic activity, and pH. Active fractions were pooled, concentrated, and dialyzed overnight against Buffer A containing 30 mM NaCl.

Step 8: Blue Sepharose Chromatography—The enzyme fraction from Step 7 was added to 0.5 ml of blue Sepharose contained in a Pasteur pipette. The column was washed with Buffer A, and the enzyme emerged in the wash. This fraction was concentrated and dialyzed against Buffer A.

A summary of the purification procedure is presented in Table I and is briefly discussed under “Results.”

Characterization of the Reaction Products

The reaction products, formed in either the forward direction (i.e. UDP-sugars) or the reverse direction (i.e. UTP and sugar 1-phosphates), were isolated by chromatography on a 1 × 20-cm column of DEAE-cellulose (HCO₃⁻) equilibrated with 10 mM NH₄HCO₃. After application of the sample, the column was washed with the same solution, and the retained products were eluted with 200 ml of a linear 10–300 mM gradient of NH₄HCO₃. Fractions were analyzed for nucleotides by measuring the absorbance at 260 nm, for radioactivity by scintillation counting, and for hexosamine by the Reissig test (8) or the Elson-Morgan assay (9). Peak fractions were pooled and concentrated on a rotary evaporator, and NH₄HCO₃ was removed by evaporation in the presence of triethylamine. The triethylamine was then removed by evaporation in the presence of methanol.

The reaction products were identified by chromatography either directly or following various treatments such as digestion with phosphodiesterase or alkaline phosphatase or acid hydrolysis. Nucleotides were identified by TLC in the following systems: polyethyleneimine plates developed in 0.2 and 0.4 M solutions of LiCl; cellulose plates in ethanol, 1 M ammonium acetate, pH 7.3 (7.3); or silica gel plates in ethanol, 1 M ammonium acetate, pH 5.0 (7.3). N-Acetyhexosamines were released from nucleotides by mild acid hydrolysis (0.1 N HCl, 100°C, 15 min) and identified by chromatography on borate-treated paper in 1-butanol/ pyridine/water (6:4:3) as described earlier by Cardini and Leloir (10).
Sugars were detected by the method of Reissig et al. (8). Deacetylation of the N-acetylhexosamines in 2 N HCl at 100 °C for 2 h gave amino sugars that were analyzed by paper chromatography in butanol/pyridine/H2O (6:4:3) and detected by the silver nitrate dip (11) or the Elson-Morgan spray (9).

Polyacrylamide Gel Electrophoresis

Native PAGE was performed as described by Laemmli (12) with an 8% gel and a discontinuous buffer system, but under nondenaturing conditions. Two samples were run in parallel. One lane was stained with Coomassie Blue to detect protein bands; the other was cut into 1-cm pieces, and each piece was crushed in 200 μl of Buffer A and incubated overnight in the cold to elute the enzyme. The gel was removed by centrifugation, and the supernatant liquid was analyzed for pyrophosphorylase activity.

SDS-PAGE was done as described (13). Prior to electrophoresis, protein samples were mixed with sample buffer (62 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.002% bromphenol blue) and heated in a boiling water bath for 5 min.

Determination of Native Molecular Mass and pi Value

The molecular mass of the enzyme was estimated from its elution profile on a calibrated 1.7 × 112-cm column of Sephacryl S-300 equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. The following proteins were run as standards: carbonic anhydrase, 29 kDa; creatine kinase, 80 kDa; lactic acid dehydrogenase, 140 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa; and thyroglobulin, 660 kDa. The isoelectric point was determined by chromatofocusing with the use of a Polybuffer Exchanger 94 column (1 × 35 cm). The column was equilibrated with 25 mM histidine HCl buffer, pH 6.5. The formation of the pH gradient and the elution of the enzyme were done using 10-fold diluted Polybuffer 74, pH 4.5, according to the Pharmacia protocol. The pyrophosphorylase was eluted at pH 5.3.

Antibody Production and Western Blotting

Polyclonal antibody against the native enzyme and against the purified 64-kDa peptide (using a total of ~200 μg of protein) was prepared in rabbits following standard procedures (14). For immunoblot analysis, proteins were electrophoretically transferred to Immobilon (15) and treated with crude antiserum at a 1:4000 dilution. Immunoprecipitation was done by incubating enzyme with crude antiserum and precipitating the complex with a mixture of protein A-Sepharose and protein G-Sepharose.

RESULTS

Purification of UDP-GlcNAc Pyrophosphorylase

UDP-HexNAc pyrophosphorylase was purified from pig liver following the procedure outlined under “Experimental Procedures.” The liver extract was first clarified by acid treatment, followed by ammonium sulfate fractionation and DEAE-cellulose chromatography. This chromatographic procedure gave a 10-fold purification, and gel filtration on a 95-cm column of Sephacryl S-300 gave another 7-fold purification. Fig. 1 presents the profiles of subsequent purification steps on pentyl-Sepharose (panel A), heparin-Sepharose (panel B), and IDA-Sepharose (panel C) columns. These three steps resulted in >60-fold purification. Thus, hydrophobic chromatography on the pentyl-Sepharose column resolved the enzyme from the bulk of inactive protein and resulted in a 12-fold increase in specific activity, while the next two steps also removed substantial amounts of protein and improved the specific activity. The total purification scheme, summarized in Table I, resulted in a 9500-fold purification of UDP-HexNAc pyrophosphorylase, with a final yield of ~2%. From 2 kg of pig liver, ~340 μg of pure protein were isolated, and this proved to be enough protein to obtain some amino acid sequence information and to prepare antibodies in a rabbit.

Properties of the Purified Pyrophosphorylase

Molecular Mass and Homogeneity of the Pyrophosphorylase—The enzyme from the final stage of purification gave a single protein band on native gels as demonstrated in Fig. 2A (lane 4). This band was eluted from the gels and assayed. It showed high UDP-GlcNAc pyrophosphorylase activity, indicat-
Purification to Homogeneity of UDP-GlcNAc Pyrophosphorylase

| Purification step | Protein | Specific activity | Purification (factor) | Yield |
|-------------------|---------|------------------|-----------------------|-------|
| 1. Purified extract | 213,840 | 0.0015 | 1 | 100 |
| 2. Ammonium sulfate | 62,775 | 0.005 | 3.4 | 98 |
| 3. DEAE-cellulose | 5083 | 0.056 | 36 | 83 |
| 4. Sephadex G-200 | 1620 | 0.110 | 71 | 67 |
| 5. Pentyl-Sepharose | 40 | 1.37 | 878 | 17 |
| 6. Heparin-Sepharose | 8.2 | 2.86 | 1836 | 7 |
| 7. IMAC column | 1.88 | 6.67 | 4278 | 6 |
| 8. Blue Sepharose | 0.34 | 14.86 | 9526 | 2 |

*IMAC, immobilized metal ion affinity chromatography.

Fig. 2. Gel electrophoresis of UDP-GlcNAc pyrophosphorylase. A shows native gels of various enzyme fractions stained with Coomassie Blue as follows: lane 1, fraction from pentyl-Sepharose; lane 2, fraction from heparin-Sepharose; lane 3, fraction from IDA-Sepharose; lane 4, fraction from blue Sepharose. B shows SDS gels of the enzyme fraction stained with Coomassie Blue at various stages of purification as follows: lane 1, crude extract; lane 2, DEAE-cellulose fraction; lane 3, pentyl-Sepharose; lane 4, heparin-Sepharose; lane 5, IDA-Sepharose; lane 6, blue Sepharose; lane 7, protein standards.

Fig. 3. Immunological cross-reactivity of UDP-GlcNAc pyrophosphorylase polypeptides with antibody against the 64-kDa polypeptide. In A, the purified pyrophosphorylase was subjected to SDS-PAGE, and the polypeptides were located by staining with Coomassie Blue (lane 1). Each of the individual polypeptides (i.e. 57-, 49-, and 49-kDa bands) was eluted from the SDS gels of the intact enzyme and subjected to a second SDS-PAGE as seen in lanes 2 (64-kDa band), 3 (57-kDa band), and 4 (49-kDa band). Molecular mass standards are shown in lane 5. In B, Western blots were done on duplicate gels of the proteins shown in A using the antibody prepared against the purified 64-kDa protein. After incubation with the antibody, the gels were treated with goat anti-rabbit IgG tagged with alkaline phosphatase.

against each of the purified protein bands. It is clear that the antibody against the 64-kDa protein reacts well with each of the protein bands, indicating that they are all closely related. These data suggest that the 57- and 49-kDa polypeptides are probably proteolytic degradation products of the 64-kDa protein and that this proteolysis occurs even in the presence of a mixture of protease inhibitors.

Antibodies against the native enzyme and the antibody prepared against the isolated 64-kDa protein were tested to determine whether they could be used to immunoprecipitate the active enzyme from solution in the presence of protein A. Thus, as shown in Fig. 4, various amounts of either antibody were incubated overnight in an ice bath with the purified enzyme preparation, and then protein A was added. After a second incubation for 1 h, the reaction mixtures were centrifuged in a microcentrifuge, and the supernatant liquids were tested for enzyme activity. It can be seen that the antibody prepared against the native enzyme was fairly effective in removing most of the enzymatic activity from the solution. The antibody prepared against the isolated 64-kDa band was also able to remove the enzymatic activity from the solution, but not quite as effectively as the antibody against the native enzyme. The control curve shows that the preimmune serum from rabbits used for the antibody preparation (or from nonimmunized animals) did not inhibit the enzymatic activity and did not remove it from solution.

Photoaffinity Labeling of UDP-HexNAc Pyrophosphorylase—Photoaffinity analogs of the UDP-sugar substrates were chemically synthesized by attaching a salicylic acid to the uridine through an allylamine to make azido-[125]salicylate-allylamine-UDP-GlcNAc ([125]ASA-UDP-GlcNAc) and azido-[125]salicylate-allylamine-UDP-GalNAc ([125]ASA-UDP-GalNAc).2 These probes proved to be useful labels for the UDP-HexNAc pyrophosphorylase as shown in Figs. 5 and 6. In these experiments, the purified and active UDP-HexNAc pyrophosphorylase was incubated for 30 s with either radiolabeled probe, and

2 Zeng, Y., Shabalin, Y., Szumilo, T., Pastuszak, I., Drake, R. R., and Elbein, A. D. (1996) Anal. Biochem., in press.
the mixture was exposed for 90 s to UV light to activate the azido group. The protein was then precipitated by the addition of 5% trichloroacetic acid; the protein was subjected to SDS-PAGE; and radiolabeled protein was detected by exposure to film (17).

As shown in lane 1 of Fig. 5, both the 64- and 57-kDa peptides from the pyrophosphorylase became labeled with 125I-ASA-UDP-GlcNAc, indicating that both proteins recognized UDP-GlcNAc as the substrate (or product). As seen in lanes 2–4, increasing amounts of unlabeled UDP-GlcNAc (0.01, 0.1, and 1 mM) effectively inhibited the labeling of both protein bands to about the same extent and in a concentration-dependent manner. The labeling of both subunits was also blocked, but not as effectively, by unlabeled UDP-GalNAc, as shown in lanes 8–10. Finally, as seen in lanes 5–7, UDP-glucose also inhibited the labeling to some extent, but it was much less effective than either UDP-GlcNAc or UDP-GalNAc.

Fig. 6 presents a comparison of the labeling of UDP-HexNAc pyrophosphorylase by either 125I-ASA-UDP-GlcNAc (lanes 1–8) or 125I-ASA-UDP-GalNAc (lanes 9–15). With this particular enzyme preparation, all three protein bands were present and became labeled by these probes, although the 57-kDa band was apparently the major protein in this particular preparation. Lane 2 demonstrates that the UDP-GlcNAc probe labels the enzyme very effectively, but the sample must be exposed to UV light in order to activate the azido group and to couple it to the protein (compare with lane 1). As previously seen in Fig. 5, 1 mM UDP-GlcNAc (lane 4) is more effective in inhibiting the labeling than is 10 mM UDP-GalNAc (lane 6) or 10 mM UDP-glucose (lane 7). Labeling was also inhibited by adding 100 mM UTP (lane 8).

The enzyme could also be labeled with the UDP-GalNAc probe, although the labeling was not as intense. The diminished labeling with this probe is probably partially due to the lower affinity of the enzyme for UDP-GalNAc, but may also be due to a lower specific activity in the UDP-GalNAc probe since this compound was more difficult to synthesize and the yield was much lower. Nevertheless, as seen in Fig. 6, when the enzyme and probe were exposed to UV light (lane 10), the three polypeptide bands became labeled and in about the same ratios as with the UDP-GlcNAc probe, but no labeling occurred in the absence of exposure to UV light (lane 9). The labeling of all three protein bands was effectively blocked by adding 10 or 100 mM UDP-GalNAc (lanes 12 and 13), 10 mM UDP-GlcNAc (lane 14), or 10 mM UDP-glucose (lane 15). These data, taken together with the antibody studies, strongly indicate that the three polypeptide bands are all related and are catalytically active subunits of UDP-HexNAc pyrophosphorylase. We propose that the native enzyme is composed of two identical 64-kDa subunits and that during the life cycle of this protein, these subunits are subjected to partial proteolysis by a (specific) protease that cleaves the 64-kDa subunits into smaller, but still catalytically active, proteins.

Stability—The purified enzyme was quite stable when stored on ice in Buffer A containing 0.05% sodium azide. Under these conditions, the half-life of the enzyme was estimated to be ~5 months. The enzyme was also stable to storage in the frozen state, and >90% of the activity still remained after 4 months of storage. The best stability was observed upon storage at pH 6.5–7.5.

Kinetic Constants—The effect of substrate concentration on the enzymatic activity and the $V_{\text{max}}$ with each substrate was determined as shown in Table II. Since the enzyme activity can be measured in the forward or reverse direction, the Michaelis-Menten constants and the $V_{\text{max}}/K_m$ values were determined in both directions with regard to activities for the synthesis or pyrophosphorylation of UDP-GlcNAc, UDP-GalNAc, or UDP-
glucose. Since the reaction in either direction has two substrates and a metal ion requirement, the calculation of the $K_m$ for one substrate was done, as much as possible, under conditions in which the concentration of the second substrate was 5–10 times its $K_m$ value. However, at high concentrations, UTP and Mg$^{2+}$ form a complex and precipitate from solution. Thus, it was not possible to use concentrations of UTP above 5 mM. In addition, radiolabeled UDP-sugars are very expensive, and this factor limits the concentrations that are reasonable to use for the UDP-[3H]GlcNAc and UDP-[3H]GalNAc determinations.

However, based on the measurements obtained (Table II), the enzyme has a much higher affinity for UDP-GlcNAc and GlcNAc-1-P than for UDP-GalNAc and GalNAc-1-P, but the $V_{max}/K_m$ values in the forward reaction are quite similar for the synthesis of either of the two UDP-sugars. It is possible that the $K_m$ values for these substrates are altered as a result of the partial proteolysis of the normal 64-kDa subunit. Future studies to isolate intact and non-proteolyzed enzyme or to clone the enzyme may help to clarify this dilemma and to determine the real role of the UDP-GalNAc activity.

pH Optimum—The pH optimum for the pyrophosphorylase was measured in the reverse direction by both the radioactive assay and a colorimetric assay as described under "Experimental Procedures." In both cases, the pH optimum was between 8.5 and 8.9. This pH profile is quite different from those described for the UDP-GlcNAc pyrophosphorylases from S. aureus and calf liver, which had a pH optimum of 7.2 (5), or that from brain, with a pH optimum of 8.0 (6).

Metal Ion Requirements—The pyrophosphorylase showed absolute requirements for a divalent cation for activity as indicated in Table III. The effectiveness of the ion activators was in the order Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$, and Ni$^{2+}$, but measurable activity was also observed with Zn$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$. Other ions such as Fe$^{3+}$, Ca$^{2+}$, and Hg$^{2+}$ were ineffective. The concentration curves of these metal ion effectors indicated that Mn$^{2+}$, Co$^{2+}$, and Mg$^{2+}$ were the most effective in the range of 0.5–1.0 mM. The Mg$^{2+}$ concentration curves were done at low (0.1–0.4 mM) and at high (1–5 mM) concentrations of UTP, and the curves were hyperbolic in all cases. It was not possible to use concentrations of UTP above 5 mM since at high Mg$^{2+}$ concentrations, the Mg$^{2+}$–UTP complex is formed and precipitates from solution. Higher concentrations of the metal ions (i.e., above 1 mM) resulted in a considerable decrease in the activation. For the UDP-GlcNAc pyrophosphorylase from brain (6), Mg$^{2+}$ was the most effective cation, followed by Co$^{2+}$ and then Mn$^{2+}$.

Substrate Specificity—The substrate specificity of the purified enzyme was examined in both the forward and reverse directions. Table IV shows that in the forward direction and at 0.5 mM sugar-1-P, the enzyme is more active with GalNAc-1-P than it is with GlcNAc-1-P, while other sugar phosphates such as Glc-1-P and Gal-1-P may act as substrates, but to a much lower degree. In the reverse direction, the pyrophosphorylase was assayed at both 0.5 and 5 mM concentrations of the UDP-sugar substrate, and the relative activities of UDP-GalNAc and UDP-glucose were compared with that of UDP-GlcNAc. Table IV shows that at low concentrations of substrate, UDP-GlcNAc is the best substrate, and UDP-GalNAc and UDP-glucose are only 91% and 54% as effective, respectively. However, at 5 mM, UDP-GalNAc is as effective as UDP-GlcNAc, while UDP-glucose is also a good substrate. Because the enzyme has a rather broad substrate specificity, we tested a number of UDP-sugars to determine whether any of them could undergo pyrophosphorylation by this enzyme. However, UDP-galactose, UDP-glucuronic acid, UDP-galacturonuronic acid, UDP-mannose, and ADP-glucose were all inactive. Other UDP-GlcNAc pyrophosphorylases such as the sheep brain enzyme were most active with UDP-GlcNAc, and some activity was observed with UDP-glucose (36%), but not with UDP-GalNAc (6). Somewhat similar results were observed with the calf liver enzyme. The enzyme from bacteria (5) acted on UDP-GlcNAc and showed only marginal activity on UDP-GalNAc (2.6%).

Effect of Inhibitors—Since the enzyme catalyzes the pyrophosphorylation of three substrates, UDP-GlcNAc, UDP-GalNAc, and UDP-Glc, it was of interest to determine how the presence of one sugar nucleotide would affect the phosphorolysis of another sugar nucleotide. In addition, if one of these nucleotides did in fact inhibit the utilization of another, it would support the specificity data that a single enzyme utilizes all of these substrates. Fig. 7 shows that the reaction with UDP-[3H]GlcNAc was inhibited by increasing concentrations of UDP-GalNAc and UDP-glucose, but not by UDP-xylene or UDP-glucuronic acid.

Tissue Distribution—To determine whether other tissues could also utilize UDP-GalNAc and GalNAc-1-P as substrates, we prepared crude cytoplasmic extracts from a variety of porcine tissues and tested them with both UDP-[3H]GlcNAc and UDP-[3H]GalNAc as substrates. The data from this experiment...
FIG. 7. Inhibition of UDP-GlcNAc pyrophosphorylase activity by various nucleotides. The pyrophosphorylation of UDP-[3H]GlcNAc was measured in the presence of various amounts of UDP-GalNAc (curve 1, ●), UDP-glucose (curve 2, △), or UDP-xylene or UDP-glucuronic acid (curve 3, ▲). Activity was measured in the reverse direction (i.e., production of GlcNAc-1-P) as indicated under "Experimental Procedures."

are presented in Table V. Since it seems likely that there is another UDP-GlcNAc pyrophosphorylase that has very low activity with UDP-GalNAc, we compared the ratio of UDP-GlcNAc to UDP-GalNAc in these various tissues. It can be seen that the utilization of these two substrates by various tissues varied considerably, but the ratio of activity with UDP-GlcNAc/UDP-GalNAc indicated that kidney and liver were the tissues that were best able to utilize the GalNAc derivatives as substrates in relation to UDP-GlcNAc activity. This is of some interest since we recently found that some animal tissues, especially liver and kidney, have a very specific kinase that phosphorylates GalNAc in the one position to form GalNAc-1-P.3 Thus, those tissues having these two enzymes may be able to utilize free GalNAc that arises from turnover of various complex carbohydrates.

Identification of the Products of the Pyrophosphorylase

The purified enzyme was incubated with [3H]UTP, Mg2+, and GlcNAc-1-P in the presence of inorganic pyrophosphatase, and the reaction was terminated after 2 h. After deproteinization, the aqueous phase was applied to a column of DE52, and sugar phosphates and UDP-sugars were separated with a gradient of NH4HCO3 as shown in Fig. 8. A peak of radioactivity, representing 79% of the total (7.9 μmol), emerged in the UDP-GlcNAc area of the column. The radioactive material was susceptible to the action of phosphodiesterase, but not to that of alkaline phosphatase. The peak was pooled and subjected to TLC in three different solvent systems as described under "Experimental Procedures." In each case, the material co-chromatographed with authentic UDP-GlcNAc and was clearly separated from GlcNAc-1-P. Mild acid hydrolysis (0.05 N HCl, 100 °C, 15 min) gave a single radioactive spot corresponding to GlcNAc on borate-treated paper in 1-butanol/pyridine/water (6:4:3). Deacetylation of the presumptive GlcNAc gave an amino sugar with the same migration as glucosamine, and ninhydrin treatment of this amino sugar gave a pentose that corresponded to arabinose.

In analogous experiments, 8.2 μmol (82%) of UDP-GalNAc and 3.8 μmol (38%) of UDP-glucose were formed from 10 μmol of either GalNAc-1-P or glucose-1-P and UTP. The character-

ization of these products was also based on the products produced by phosphodiesterase, mild acid hydrolysis, and other treatments.

The products of pyrophosphorylation, i.e. UTP and HexNAc-1-P, were also identified in the following way. UTP was identified by its mobility on TLC plates as described under "Experimental Procedures" as well as by its UV spectrum and its utilization by diphosphonucleotide kinase. GlcNAc-1-P and GalNAc-1-P were identified by their susceptibility to alkaline phosphatase and the formation of the appropriate N-acetylhexosamine following this treatment.

No UDP-GlcNAc (GalNAc) 4-epimerase activity could be detected in the purified enzyme preparation. Thus, no GalNAc (or UDP-GalNAc) was detected when UTP and GlcNAc-1-P were incubated with the purified enzyme, even though UDP-GlcNAc was rapidly produced and readily detected.

DISCUSSION

UDP-GlcNAc pyrophosphorylase is an important enzyme in eucaryotic cells because it is responsible for the formation of UDP-GlcNAc, which is an essential precursor for the GlcNAc residues in N-linked oligosaccharides (18), proteoglycans (19), glycolipids (20), mucins (21), O-linked oligosaccharides (22), and O-linked GlcNAc (4). In addition, it is an essential component of the cell walls of yeast and fungi (23) and is also a key component (or precursor) of the peptidoglycan of many procaryotic cells (24). In spite of the great diversity of this sugar, there have been only sparse reports on the purification of this key enzyme, and none of these purifications has resulted in homogeneous enzyme.

In this study, we have been able to obtain a single protein band of UDP-GlcNAc pyrophosphorylase on native gels, and this band gave rise to two or three protein bands on SDS-PAGE. Since the apparent molecular mass of the native enzyme is ~125 kDa based on gel filtration and since the major subunits appear to have molecular masses of 64 and 57 kDa on SDS gels, we initially assumed that the enzyme was a heterodimer. However, since both of these polypeptides (and the third smaller polypeptide) show strong cross-reactivity with antibody prepared against the homogeneous 64-kDa protein and since each of the polypeptides becomes labeled with the UDP-GlcNAc and UDP-GalNAc photoaffinity labels, it seems most likely that all three polypeptides are derived from the same gene product and that the smaller polypeptides are the result of limited proteolysis of the 64-kDa band. Thus, UDP-GlcNAc (GalNAc) pyrophosphorylase is probably a homodimer of two 64-kDa subunits.

Interestingly enough, the UDP-GlcNAc pyrophosphorylase described here also works quite well with UDP-GalNAc when this substrate is present at millimolar concentrations. While

TABLE V

| Tissue assayed | Enzymatic activity | UDP-GlcNAc/UDP-GalNAc activity ratio | cpm/mg protein |
|---------------|-------------------|------------------------------------|----------------|
| Kidney        | 548               | 248                                | 2.2            |
| Liver         | 4144              | 1670                               | 2.5            |
| Lung          | 833               | 136                                | 6.1            |
| Pancreas      | 11,670            | 2572                               | 4.5            |
| Spleen        | 716               | 164                                | 4.4            |
| Aorta         | 1170              | 132                                | 8.9            |
| Brain         | 975               | 213                                | 4.6            |
| Heart         | 560               | 160                                | 3.5            |

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this concentration of UDP-GalNAc may not be physiologically significant, this enzyme could still be involved in GalNAc activation for the following reasons. 1) Since the purified enzyme appears to have undergone considerable proteolysis to give smaller catalytically active polypeptides, it is conceivable that the affinity of these altered enzyme fragments for the substrates is much lower than that of the native enzyme. 2) Since we recently have identified and purified a new GalNAc kinase from the cytosolic fraction of pig liver, it seems possible that this kinase and the pyrophosphorylase are loosely held together in a complex such as has been suggested for enzymes of the citric acid cycle (25, 26). If so, it is possible that the concentration of GalNAc-1-P in the complex might be much higher that the reported concentrations for sugar phosphates (0.5 mM or less) in the cytoplasm. Under such conditions, UDP-GalNAc pyrophosphorylase could be exposed to higher concentrations of GalNAc-1-P than found throughout the cytoplasm.

Using the purified enzyme, K_m values were determined for the various substrates in the forward and reverse directions. As indicated above, these values were somewhat higher than expected (2 mM for most substrates) and raise questions about the physiological significance of some of the activities such as the formation of UDP-GalNAc. While the explanation for these high K_m values is not clear, as suggested above, it may be that the smaller polypeptides (e.g. 57 and 49 kDa) have a lower affinity for the various substrates than the native 64-kDa protein. Fortunately, at this stage, we do not have a suitable method to remove the smaller polypeptides, nor have we been able to inhibit their formation by using a mixture of protease inhibitors during the purification procedure.

The current literature indicates that the formation of UDP-GalNAc involves the action of a 4-epimerase on UDP-GlcNAc to produce UDP-GalNAc (27). In fact, a mutant Chinese hamster ovary cell line has been isolated that is missing the UDP-GlcNAc:UDP-GalNAc 4-epimerase, and this cell line cannot produce O-linked oligosaccharides (16). Thus, in those cells, it appears that epimerization of UDP-GlcNAc is necessary for formation of UDP-GalNAc. However, based on our recent findings of a highly specific kinase that produces GalNAc-1-P and the enzyme reported here, it seems likely that kidney and liver have an alternative pathway to reutilize GalNAc with the formation of UDP-GalNAc. In any case, UDP-GalNAc (GalNAc) pyrophosphorylase should be a useful tool to make a UDP-GalNAc photoaffinity probe, and such a probe should be valuable for the identification, quantification, and purification of GalNAc-transferases.

FIG. 8. Isolation and Identification of the reaction products as UDP-GlcNAc. The enzyme was incubated with [3H]UTP, Mg^2+, and GlcNAc-1-P for up to 2 h in the presence of inorganic pyrophosphatase, and the reaction mixtures were applied to columns of DE52. Sugar phosphates and UDP-sugars were separated with a gradient of NH_4HCO_3. Fractions were analyzed for radioactivity (---), for GlcNAc by a colorimetric assay (C), and for nucleotide content (O). The upper panel is a control without incubation, while the lower panel is following a 2-h incubation. The numbers refer to the elution positions of standards, i.e. 1 = GlcNAc-1-P, 2 = UDP-GlcNAc, and 3 = UTP.

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J. Biol. Chem. 1996, 271:13147-13154.
doi: 10.1074/jbc.271.22.13147

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