Rat liver glucosidase II, an endoplasmic reticulum hydrolase involved in the biosynthesis of the N-linked class of glycoproteins, has been purified in good yield to a state approaching homogeneity. The purified enzyme hydrolyzes p-nitrophenyl-a-D-glucopyranoside, maltose, and the precursor oligosaccharides glucose₆, mannose₆, N-acetylglucosamine, but it does not act on glucos₂, mannose₂, N-acetylglucosamine or p-nitrophenyl-β-D-glucopyranoside. The ratio of the rate at which glucose is released from p-nitrophenyl-a-D-glucopyranoside to that from glucose₆, mannose₆, N-acetylglucosamine or glucose, mannose, N-acetylglucosamine remains constant throughout the 8-step purification procedure; thus it appears that a single enzyme is responsible for the activities toward both the artificial and oligosaccharide substrates. The fact that the enzyme cleaves both of the inner 1,3-linked glucosyl residues from the precursor oligosaccharides supports the view that they are linked in the α-configuration. The pH dependence of enzymatic activity is quite similar for different substrates, showing a broad optimum between pH 6 and 7.5. Activity toward p-nitrophenyl-a-D-glucopyranoside is enhanced by 12 mm 2-deoxy-D-glucose (260-300% activation) and 25 mm mannose (150% activation), but these two compounds inhibit the action of the enzyme toward the precursor oligosaccharides. By isoelectrofocusing the purified enzyme exhibits one form, which has a pI of 3.5-3.8. Reductive polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicates that glucosidase II has a subunit molecular weight of 65,000. Ferguson plot analysis of the behavior of native enzyme in polyacrylamide gels indicates that it is a 282,000-dalton tetramer. Gel filtration gives a molecular weight of 288,000. Several lines of evidence indicate that the enzyme is a glycoprotein.

Following the en bloc transfer of the Glc₄Man₁₉GlcNAc₂ precursor oligosaccharide (1) from its dolichol pyrophosphoryl derivative to nascent polypeptide chains in the biosynthesis of the N-linked class of glycoproteins (2), the three terminal glucosyl residues are sequentially removed (3). Radiolabeling studies have indicated that the enzymatic trimming of this "triglucosyl cap" is very rapid and occurs within the endoplasmic reticulum before further modification of the oligosaccharide, such as the excision of several mannose residues, occurs (4-6). Glucosidase activities which act on the precursor oligosaccharides have been observed in many different cell types (4-19); however, these investigations have generally centered upon the trimming of the oligosaccharides rather than upon the enzymes which effect this processing. Nonetheless, it is clear that there are at least two different glucosidases responsible for trimming the oligosaccharide (7, 8, 10-14, 18). One glucosidase activity first releases the terminal 1,2-linked glucose (glucosidase I); a second glucosidase activity then releases the remaining inner two 1,3-linked glucose residues (glucosidase II). The glucosidase II activity has been only partially purified from rat (7, 14) or calf (10) liver and has not been extensively characterized. The partially purified glucosidase II activity was directly associated with p-nitrophenyl-a-D-glucopyranoside; both activities appeared to be due to the same neutral glycosidase.

Our work with the rat liver neutral microsomal a-D-glucosidase, originally reported by Lejeune et al. (20), began shortly after dolichol-linked precursor oligosaccharides were reported to contain glucose (21-23). Since a specific function for this membrane-bound glucosidase activity was unknown, we postulated that it was the enzyme responsible for excising glucose residues from the precursor oligosaccharide after its transfer to protein. By utilizing p-nitrophenyl-a-D-glucopyranoside as a substrate, we have purified this neutral a-glucosidase to a state approaching homogeneity. The recent availability of glycosylated precursor oligosaccharides has permitted us to demonstrate that the pNP-a-glucosidase¹ and glucosidase II activities are expressed by the same enzyme. A preliminary report of this work has been previously presented (24).

EXPERIMENTAL PROCEDURES

Materials

Male Wistar rats (150 g) were obtained from Harlan Industries, Inc. Chemicals were obtained from the following sources: all p-nitrophenyl glycosides, 4-methylumbelliferyl-a-D-glucoside, Tris base, TES, Triton X-100, 2-mercaptoethanol, p-chloromercuribenzenesulfonate, maltose, glucose, α-methylmannoside, 2-deoxy-D-glucose, bovine serum albumin, ovalbumin, β-lactalbumin, phenylmethylsulfonyl fluoride-treated trypsinogen, transferrin, aldolase, carbonic anhydrase, ceruloplasmin, pepsin, glucose-6-phosphate dehydrogenase, hexokinase, affinity-purified Clostridium perfringens neuraminidase, and octyl-agarose were from Sigma; Bio-Gel P-4 (-400 mesh), hydroxyapatite (Bio-Gel HTP powder), sucrose, BioLyte pH 3-10 Ampholines, acrylamide, and other electrophoresis chemicals were from Bio-Rad; fluorcamine (Fluram) was from Pierce Chemical Co.; SDS was from British Drug House; phosphorylase b, catalase, trypsin

¹ The abbreviations used are: pNP-a-glucosidase, p-nitrophenyl-a-D-glucopyranosidase; TES, N-(tris[hydroxymethyl]methyl-2-amino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; NP, nitrophenyl.
inhibitor, p-lactamase, Sephacryl S-200 and Sephacryl S-300 were from Pharmacia; Whatman DIAE-cellulose (DE-52) was from Fisher; UDP-[3H]glucose (9.2 mCi/µmol), [3H]mannose, and Aquosol scintillation fluid were from New England Nuclear; purified endo-β-N-acetylglucosaminidase H (Streptomyces griseus) and endo-β-N-acetylglucosaminidase D (Diplococcus pneumoniae) were from Miles Laboratories, Inc.; purified Vibrio cholerae neuraminidase was from Calbiochem-Behring, and Conduitol B and N,N-diethyldithiocarbamic acid were generous gifts of Dr. Norman Radin (University of Michigan, Ann Arbor) and Dr. R. G. Sommerville (Edinburgh Pharmaceutical Industries, Edinburgh), respectively. All other chemicals were obtained commercially and were of the highest purity available.

Preparation of Sizing Resins and Hydroxylapatite

Before use, Sephacryl S-200 and S-300 resins were exhaustively washed with 1 ml/mg solution of ovalbumin in 10 mM phosphate, pH 7.0. Periodically, sizing columns were rewash with ovalbumin. This pretreatment significantly improved the yield of glucosidase II.

Analytical Procedures

The standard assay of p-nitrophenyl-α-D-glucosidase activity was performed by incubating enzyme in 0.5 ml of 50 mM TES-NaOH buffer, pH 6.8, containing 4 mM of substrate at 37 °C for 30 to 60 min. The incubation was terminated by adding 1.0 ml of 0.64% ethylene-diamine, pH 10.7, and the absorbance at 400 nm was determined. A simple modification of this assay was used when the presence of a non-enzymatic reaction was anticipated. In this modified procedure, the reaction was terminated with 0.5 ml of ice-cold 10% trichloroacetic acid, mixed by vortexing, and then centrifuged at 1500 × g for 10 min. A 0.5-ml aliquot of the supernatant solution was carefully withdrawn and mixed with 1.0 ml of 0.64% ethylenediamine and 1.50 ml of 2 M NaOH. In both assay procedures, 1 p-nitrophenyl-α-D-glucosidase unit was defined as that amount of enzyme required to release 1 µmol of p-nitrophenol per hr. The standard assay for 4-methylumbelliferyl-α-D-glucosidase activity was performed in the same manner, except that 1 mM substrate was used.

The cleavage of maltose by glucosidase II was assayed through the use of maltokinase and glucose-6-phosphate dehydrogenase. The production of NADPH from NADP was monitored either by the increase in 4-methylumbelliferyl-[3H]glucosidase activity only in terms of the percentage of [3H]glucose label released in a given amount of time, since neither the chemical quantitation of the amount of oligosaccharide substrate present nor the amount of glucose released was possible. In a manner analogous to past reports (7, 8, 10, 12—15), 1 unit of glucosidase II activity was defined as that amount of enzyme that released 1% of the [3H]glucose from the designated precursor oligosaccharide in 1 h.

Bradford’s “microprotein” dye-binding assay (25) was used to determine protein in solutions containing Ammonophiles or Tris buffer. However, throughout the purification steps in which phosphate rather than Tris buffers were used (Steps I—VIII of the procedure detailed below), protein was determined with fluorescamine by the procedure of Anderson and Desnick (26).

Analytical Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed using the discontinuous system of Davis (27). Glucosidase activity was visualized in the following manner. Gels were incubated in 100 mM phosphate buffer, pH 6.5, for 15 min at 37 °C. This solution was decanted and replaced by a solution of 100 mM phosphate, pH 6.8, containing 0.5 mM 4-methylumbelliferyl-β-D-glucopyranoside and 5 mM 2-mercaptoethanol. Within 15 to 30 min enzyme activity could be observed as a fluorescent band under ultraviolet illumination. The released 4-methylumbelliferone was washed from the gels by incubating overnight in 20% methanol at 37 °C with shaking. The gels were then stained for protein.

SDS-polyacrylamide gel electrophoresis was performed under reducing conditions according to the method of Laemmli (28). Prior to application to the gels, samples were dialyzed against two changes of 100 volumes of 0.2% SDS, lyophilized to dryness, and then resuspended in 0.30 ml of 50 mM Tris/HC1, pH 6.6, containing 750 mM 2-mercaptoethanol, 0.2% SDS, and 10% glycerol. After incubation at 100 °C for 5 min, the sample was layered on the stacking gel. Gels were stained for protein with 0.5% Coomassie blue in 20% methanol/10% acetic acid and destained in 10% methanol/7% acetic acid. Staining for glycoprotein was accomplished using the periodic acid–Schiff’s base technique according to Kapitany and Zebrowski (29).

Isoelectrofocusing

Analytical isoelectrofocusing was carried out in 3.5% polyacryl-

amide gels containing 0.1% Triton X-100 and a 1% (w/v) final concentration of Biotyde pH 3—10 Ammonophiles. The upper gel reservoir was 200 mM NaOH, and the lower reservoir was 150 mM phosphoric acid.

Preparative isoelectrofocusing was performed in a 110-ml capacity LKB column, with a 10—65% (w/v) sucrose density gradient employing 2.5% (v/v) pH 3—10 Ammonophiles. In either gels or density gradients, isoelectrofocusing was performed following the instructions given in LKB technical manual number 8100, Appendix I. A maximum of 2 watts of power was applied to 12 polyacrylamide gels, and a maximum of 5 watts was applied to the column.

Molecular Weight Determinations

The oligomeric molecular weight of glucosidase II was determined by polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (30) using the Davis system (27) and also by molecular sizing on a calibrated column of Sephacryl S-300. The subunit molecular weight of the enzyme was determined by SDS-polyacrylamide electrophoresis according to the method of Laemmli (28). All standard plots of M versus relative mobility (or V) were fitted to the experimental data by the method of least squares.

Preparation of [3H]Glucose-labeled Precursor Oligosaccharide

Microsomal membranes were prepared from commercially ob-
tained frozen chicken liver for use in the in vitro oligosaccharide labeling system of Grinna and Robbins (7). Following incubation of the membranes (20 mg of protein/ml) with 400 µM MnCl2, 16 µM UDP-[3H]glucosamine, in the presence of 0.5 mM 2-mercaptoethanol, 0.2% SDS, and 10% glycerol. After incubation at 100 °C for 5 min, the sample was layered on the stacking gel. Gels were stained for protein with 0.5% Coomassie blue in 20% methanol/10% acetic acid and destained in 10% methanol/7% acetic acid. Staining for glycoprotein was accomplished using the periodic acid–Schiff’s base technique according to Kapitany and Zebrowski (29).

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GlcManGlcNAc comprising most of the remaining radioactivity recovered. There was some contamination of the GlcManGlcNAc component with GlcManGlcNAc (about 15% contamination).

Preparation of Subcellular Fractions for the Purification of Glucosidase II

20 male Wistar rats (150 g) were fasted for 36 h prior to sacrifice by decapitation. The livers were minced and washed extensively in 0.25 M sucrose containing 10 mM Tris-Cl, pH 8.0, and 5 mM 2-mercaptoethanol before they were blotted and weighed. The livers were suspended in 4 volumes of the same sucrose buffer and carefully homogenized in a Waring blender (ten 4-s pulses, separated by 20-s pauses to minimize foaming). The nuclear fraction was prepared by centrifugation at 1500 × g for 10 min, and the fraction was homogenized and centrifuged again. The supernatant solution was centrifuged at 8000 × g for 15 min, and the pellet was homogenized and centrifuged again to yield the mitochondrial-lysosomal fraction. This procedure minimizes the loss of glucosidase II to this fraction. The postmitochondrial-lysosomal supernatant solution was diluted with the sucrose buffer to a final ratio of 20 ml of g per liver and made 10 mM in freshly prepared CaCl₂ in order to precipitate microsomes (31).

The suspension was made 1% (v/v) in Triton X-100 by dropwise addition of detergent with stirring. Stirring was continued for 2 h, and the mixture was then centrifuged at 78,000 × g for 4 h. The supernatant solution routinely contained 95-100% protein. This mixture was brought to room temperature, stirred for 20% of the activity recovered from the column, it often was present in only trace amounts.) The major component was pooled by combining column fractions with highest enzymatic activity so that the pool possessed a specific activity of at least 11 units/mg.

Step V: Chromatography on DEAE-Cellulose—The DE-52 eluate was diluted to 130% of its volume with 10 mM phosphate, pH 7.0, and applied to a column of Bio-Rad HTD hydroxylapatite (1 × 4 cm). The enzyme eluted as a single symmetrical peak at approximately 200 mM phosphate in a 200-ml linear phosphate gradient (10 to 400 mM), pH 7.0. The column fractions were combined to give a specific activity of at least 30 units/mg for the pool.

Step VII: Gel Filtration on Sephacryl S-300—The pooled eluate from the hydroxylapatite column was concentrated as before to 3 ml, made 20% in glycerol, and applied to a column (1 × 45 cm) of Sephacryl S-300. The same column buffer was used as that used with the S-200 gel filtration step (Fig. 1A), and the flow rate was adjusted to 4 ml/h. The enzyme eluted as a single component (Vᵣ/Vₘ = 4.16 and Vᵣ/Vₘ = 0.433). This peak contained 70 to 90% of the applied activity. The pool of the fractions containing most of the enzyme activity had a specific activity of 45 to 65 units/mg.

Table I  The purification of glucosidase II

| Purification step | Glucosidase II activity | Protein content | Specific activity | Purification activity | Yield |
|------------------|------------------------|----------------|------------------|-----------------------|-------|
|                  | pNP-glucosidase units | mg             | units/mg         | (10⁵)                | %     |
| I. Triton extract| 406                    | 1790           | 0.23             | 1                     | 100   |
| II. Batch hydroxylapatite | 350                   | 353             | 0.86             | 3.8                   | 75    |
| III. ConcanaVill A-Sepharose 4B | 34 (12.5)            | 69              | 8.9              | 35.4                  | (105) |
| IV. Sephacryl S-300 | 13.6 (13.4)           | 69              | 29.5             | (105)                 | 31    |
| V. DEA5-cellulose | 58                     | 3.18            | 18.8             | 80.1                  | 14.3  |
| VI. Hydroxylapatite column | 63                   | 1.81            | 30.0             | 130                   | 25.5  |
| VII. Sephacryl S-300 | 51.4                   | 1.15            | 44.7             | 194                   | 12.7  |
| VIII. Hydroxylapatite column | 51.0               | 0.640           | 79.5             | 344                   | 12.6  |

"The hydrolysis of pNP-α-D-glucopyranoside was assayed as described under "Experimental Procedures."

"Protein was determined with fluorescamine as described under "Experimental Procedures."

"Activation by α-methylmannoside during elution from this column results in the apparent high yield and high purification from Step III. For this reason, these values are given in parentheses.

"During Step IV, a minor form is sometimes observed. This activity may represent artifactual modification of glucosidase II, and so its values are reported in parentheses.
Purification and Characterization of Glucosidase II

Evidence that Glucosidase II and the Neutral pNP-α-Glucosidase Are Identical—The purified enzyme was analyzed by electrophoresis on 6% polyacrylamide tube gels (see under "Experimental Procedures"). When enzymatic activity was visualized using 4-methylumbelliferyl-α-D-glucoside, only a discrete doublet was observed (Fig. 2A). Subsequent staining of the same or duplicate gels for protein with Coomassie blue demonstrated one closely spaced doublet identical in position and appearance with the activity band. In 5 to 10% gels, the protein and activity bands always comigrated exactly; furthermore, the spacing of the doublet did not change. Overloaded gels gave the same results. When samples from different parts of the pNP-α-glucosidase activity peak obtained with the hydroxylapatite column (Step VIII; Fig. 1B, fractions 19, 21, and 25) were analyzed by electrophoresis, the same discrete doublet of activity and protein banding was observed. The two portions of the doublet were still approximately equivalent in intensity. The same results were obtained with samples from the Step IV Sephacryl S-200 column (Fig. 1A, fractions 65, 69, 71, and 77).

The purity of the enzyme was further investigated by reductive SDS-polyacrylamide electrophoresis in the Laemmli system (see under “Experimental Procedures”). Under these conditions, the enzyme migrated as one major band on 8 and 10% gels (Fig. 2B). There were minor bands present on overloaded gels that migrated faster than the major bands; these contaminants constituted about 10% of the total protein seen on the gels (Fig. 2B, first gel). These bands may be derived from the glucosidase II subunit, or they may truly represent contaminants that were not observed on the overloaded native gels. The major band was always rather broad, and it is

Fig. 1. Behavior of glucosidase II in gel filtration with Sephacryl S-200 and in chromatography on hydroxylapatite. A, elution of pNP-α-glucosidase activity from a column (2.5 × 96 cm) of Sephacryl S-200 using 10 mM phosphate, pH 7.0, 200 mM NaCl, 0.1% Triton X-100, and 5 mM 2-mercaptoethanol as elution buffer (Step IV of the purification procedure). The fraction size was 3.2 ml. Enzymatic activity (○) and protein content (□) were determined as described in the legend to Table I. The determined void volume (Vv) is indicated. B, elution profile of enzymatic activity from the final step of the purification procedure, the second hydroxylapatite column (Step VIII). The fraction size was 2.5 ml. Conductivity (∇) was monitored in order to follow the linear (10 to 400 mM) phosphate gradient, pH 7.0. Enzymatic activity (○) and protein (□) eluted at approximately 200 mM phosphate.

RESULTS

Subcellular Distribution of Neutral Glucosidase

Lejeune et al. (20), using maltose as substrate, reported that rat liver contains both membrane-bound and soluble neutral α-glucosidase activities. Using pNP-α-glucosidase as substrate, we found that the [105,000 × g] microsomal fraction contained 45% of the activity in the homogenate; the remainder was present in the cytosol. That the microsomal enzyme was present in the endoplasmic reticulum rather than in Golgi or plasma membranes was strongly suggested by the work of Dewald and Touster (32). Their experimental results were confirmed in the present study (data not shown). Moreover, the amount of neutral pNP-α-glucosidase activity obtained from a highly purified Golgi fraction (33) was less than 1% of the amount found in washed microsomes and had a specific activity of 0.040 unit/mg, approximately one-fourth the specific activity obtained with the endoplasmic reticulum fraction. Therefore, a washed microsomal fraction was used as the source of the enzyme for purification procedures.

Purification of Glucosidase II

Table I shows the results of a typical purification experiment (see under "Experimental Procedures"). Variability in the final two steps (VII and VIII) sometimes resulted in a lower extent of purification and a lower yield. The best results were obtained when the purification was accomplished rapidly. Unless otherwise indicated, the studies reported below utilized enzyme purified through Step VIII.

Characteristics of Glucosidase II

The degree of purification of glucosidase II obtained through Step VIII is indicated in Table I. In chromatography on hydroxylapatite (Fig. 1A), the homogeneous fraction eluted as a single symmetrical peak. The activity and protein elution profiles followed each other closely (Fig. 1B). The specific activity across the peak was 76 to 80 units/mg and after pooling and concentration was 80 units/mg. This constituted a 344-fold purification over the microsomal Triton extract and corresponded to a 2650-fold purification of the activity in the homogenate; the remainder was confirmed in the present study (data not shown). Moreover, the amount of neutral pNP-α-glucosidase activity obtained from a highly purified Golgi fraction (33) was less than 1% of the amount found in washed microsomes and had a specific activity of 0.040 unit/mg, approximately one-fourth the specific activity obtained with the endoplasmic reticulum fraction. Therefore, a washed microsomal fraction was used as the source of the enzyme for purification procedures.

Fig. 2. Electrophoretic behavior of purified glucosidase II. A, the direct correlation between the activity stain and protein stain bands of purified enzyme on 6 and 8% Davis system nondenaturing polyacrylamide gels. Enzymatic activity was visualized through the cleavage of the 4-methylumbelliferyl-α-D-glucoside substrate, and protein was stained with Coomassie blue. Further details are available under “Experimental Procedures.” The figures in A are drawn from the original gels; no other protein or activity bands were detected, even with severely overloaded gels. B, the results of reducing SDS Laemmli system electrophoresis of different preparations of purified glucosidase II. In each case, the most prominent band constitutes 90% or more of the total protein detected. It is more common to see the low molecular weight contaminants of the type visible in the first (8%) gel, but occasionally higher molecular weight contaminants, such as those in the second (10%) gel, were observed.
possible that it was an unresolved doublet (Fig. 2B, second gel).

The highly purified enzyme efficiently catalyzed the release of glucose from Glc3Man9GlcNAc and Glc3Man9GlcNAc, but not from Glc3Man3GlcNAc. Table II demonstrates the ability of the enzyme to cleave these oligosaccharides at different stages of the purification scheme. In general, 70 to 80% of the glucose was released from Glc3Man9GlcNAc. The ability to release glucose from Glc3Man9GlcNAc (glucosidase I activity) was lost during the Sephacryl S-200 sizing step (Step IV).

Due to incomplete separation of Glc3Man9GlcNAc and Glc3Man9GlcNAc during their preparation (see under "Experimental Procedures"), the Glc3Man9GlcNAc used in these experiments was known to contain a 10-15% contamination of Glc3Man9GlcNAc; this probably accounted for the low amounts (less than 5-6%) of glucose released from Glc3Man9GlcNAc during the latter stages of enzyme purification. The release of about 80% of the glucose label from the Glc3Man9GlcNAc and Glc3Man9GlcNAc agreed very well with previous reports (7, 8, 10, 13, 14).

Table II also presents data pertaining to the ratio between pNP-α-glucosidase activity and the cleavage of glucose from Glc3Man9GlcNAc throughout the purification procedure. It is apparent that 1 unit of pNP-α-glucosidase activity possessed about 3000 "Glc" units of activity throughout the purification and about 6000 "Glc" units of activity. Purified glucosidase II releases glucose from Glc3Man9GlcNAc at a higher rate than from Glc3Man9GlcNAc; this finding is in agreement with observations in previous reports (7, 10, 13, 14). More importantly, since the ratio of activity toward pNP-α-glucoside and the oligosaccharides remains essentially constant throughout the purification, it appears that a single enzyme is responsible for both types of activity. Enzyme purified 2000-fold by an alternate purification scheme involving sucrose density gradients (Fig. 3A) gave a value of 288,000 pNP-glucosidase unit using the standard assay conditions.

**Table II**

Cleavage of the Glc3Man9GlcNAc precursor oligosaccharides by glucosidase II at different stages of the purification procedure

| Enzyme source | Maximal release of [%H]glucose from Glc3Man9GlcNAc oligosaccharides | Rate of release of [%H]glucose from Glc3Man9GlcNAc oligosaccharides by glucosidase II* | Calculated Glyc and Glc units per 1.0 pNP-glucosidase unit of glucosidase II† |
|---------------|---------------------------------------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| 1% Triton extract of washed microsomes (Step I) | 69 | 78 | 75 | 29.5 | 13.2 | 5000 | 2650 |
| Elution from concanavalin A-Sepharose 4B (Step III) | 68 | 76 | 76 | ND | ND | ND | ND |
| Major component from Sephacryl S-300 | Trace | 77 | 75 | 31.0 | 14.8 | 6200 | 2850 |
| The purified enzyme from the final hydroxyapatite column (Step VIII) | Trace | 80 | 78 | 30.5 | 15.2 | 6100 | 3000 |

*Expressed as the percentage of total radioactivity released by 0.02 pNP-glucosidase unit of glucosidase II using the standard assay conditions described under "Experimental Procedures" except that incubation time was extended to 6 h.

†The [%H]glucose-labeled precursor oligosaccharides were prepared from chicken liver microsomes as described under "Experimental Procedures."
absence of their inhibitors caused basic PI shifts of approximately 1.3 pH units. These shifts were greatly reduced when the appropriate inhibitors were present. On the other hand, endo-β-N-acetylglucosaminidase (0.01 unit, 50 mM citrate, pH 5.5) had no effect on the PI of glucosidase II. The results suggested that modification of sialic acid-containing oligosaccharides may be largely responsible for the PI shifts observed.

A brief investigation of the cytosolic neutral α-glucosidase activity revealed a major component with a PI of 5.2 and a minor component with a PI of 3.8. The minor component may represent microsomal α-glucosidase lost to the cytosol during preparation of washed microsomes, whereas the major component may be the normal cytosolic enzyme previously reported (20).

**Stability of Glucosidase II**—The enzyme was stable (greater than 85% retention of activity for at least 1 week) between pH 6.0 and 8.0 only in the presence of 5 mM 2-mercaptoethanol. Phosphate buffer stabilized the enzyme better than borate, barbital, Tris, or TES buffers.

The 1% Triton X-100 extract of washed microsomes was subjected to heat treatment in 50 mM phosphate, pH 7.0, and 5 mM 2-mercaptoethanol in order to study the thermal inactivation of the enzymatic activity and also to discern whether there might be more than one pNP-α-glucosidase present in the extract. The enzyme was completely stable for at least 2 h at 36°C but was slowly inactivated at 40°C. The apparent first order inactivation kinetics at 44°C indicated that there was present only one major enzymatic form capable of cleav-

**Fig. 3. Determination of oligomeric molecular weight of glucosidase II by polyacrylamide gel electrophoresis and gel filtration on Sephacryl S-300.** A, a typical molecular size estimation by Ferguson plot analysis (30). Gel concentrations of 5, 6, 7, 8, and 9% were used with the Davis system, and the slope (Ka) of the plot of R vs %T for each protein was then plotted versus the known molecular weights. The standard proteins were as follows: 1-apolipoprotein (M₉ = 445,000), 2-preputial β-glucuronidase (M₉ = 280,000), 3-aidolase (M₉ = 160,000), 4-ceruloplasmin (M₉ = 125,000), 5-tranferrin (M₉ = 80,000), 6-bovine serum albumin (M₉ = 67,000), and 7-purified glucosidase II. In the experiment shown, the estimated molecular weight was 305,000. B, a typical molecular size estimation using a calibrated Sephacryl S-300 sizing column. The standards were: 1-thyroglobulin dimer (M₉ = 662,000), 2-apolipoprotein (M₉ = 445,000), 3-preputial β-glucuronidase (M₉ = 280,000), 4-catalase (M₉ = 250,000), 5-lactate dehydrogenase (M₉ = 140,000), and 6-bovine serum albumin (M₉ = 67,000). The arrow indicates the elution volume of purified glucosidase II; the estimated molecular weight from this experiment was 282,000. The elution buffer was the same as that used for the Sephacryl S-200 column (Fig. 2A).

**Fig. 4. Determination of the subunit molecular weight of glucosidase II by SDS-polyacrylamide gel electrophoresis.** Purified glucosidase II was analyzed on reducing SDS-polyacrylamide gels using the discontinuous system of Laemmli (28). The standards were: 1-phosphorylase b subunit (M₉ = 94,000), 2-bovine serum albumin (M₉ = 67,000), 3-catalase subunit (M₉ = 60,000), 4-ovalbumin (M₉ = 43,000), 5-carbonic anhydrase (M₉ = 29,000), 6-trypsin inhibitor (M₉ = 20,500), 7-ferritin subunit (M₉ = 18,500), and 8-α-lactalbumin (M₉ = 14,500).

**Fig. 5. Isoelectrofocusing of crude and purified glucosidase II.** Sucrose density gradient (10 to 65%, w/v) isoelectrofocusing was performed with a microsomal 1% (v/v) Triton X-100 extract of microsomes (A) or purified glucosidase II (B) as described under "Experimental Procedures." pNP-α-Glucosidase (pNP-glucosidase) activity is presented as units/fraction (●) and protein as A₂₈₀/fraction (○) or mg/fraction (△). X indicates pH. Fraction size was 1.5 ml. G₃₅₄N, GlcManGlcNAc.
ing pNP-α-glucoside. The heat inactivation of a 2-week-old 1% Triton extract was considerably different. Although this extract had retained 90% of its original activity, the enzymatic activity was rapidly inactivated at 46 °C.

The enzyme was best stored for several weeks by quick-freezing in liquid nitrogen in a solution of 100 mM phosphate buffer, pH 7.0, 100 mM maltose, and 5 mM 2-mercaptoethanol. For several days highly concentrated enzyme (0.5 mg/ml) was relatively stable at 4–6 °C in a solution composed of 10% glycerol, 100 mM phosphate, pH 7.0, and 5 mM 2-mercaptoethanol.

Enzymatic Properties of Isolated Glucosidase II

**pH Dependence**—As shown in Fig. 6A, the observed pH dependence of glucosidase II activity toward pNP-α-glucoside was nearly identical with that reported initially by Lejeune et al. (20) for the hydrolysis of maltose. The pH dependence for the hydrolysis of 4-methylumbelliferyl-α-D-glucopyranoside was essentially the same (data not shown). With the precursor oligosaccharides as substrates, the pH activity curves were more sharply defined than those with the above three substrates (Fig. 6A). With Glc₃Man₂GlcNAc, the optimal pH was 6.6, and with Glc₃Man₃GlcNAc it was 7.0. It should be noted that purified enzyme showed no activity in the pH range in which the lysosomal acid α-glucosidase is active (pH 3.0 to 5.0, with the optimum at pH 4.5 (37)).

**Time Course**—The release of glucose from Glc₃Man₂GlcNAc by glucosidase II reaches a maximum of approximately 80% (Fig. 6B), in agreement with the data in Table II, and is linear for 35 to 40 min. Glucose release from Glc₃Man₃GlcNAc attained the same maximum, but the rate of release was somewhat slower.

**Kinetics: Activators and Inhibitors**—The cleavage of pNP-Glc by purified glucosidase II follows Michaelis-Menten kinetics. Double reciprocal plots of the data yielded a Kₘ of 0.85 mM and Vₘₐₓ of 0.090 μmol/h/μg of enzyme (Fig. 7). The cleavage of maltose also followed Michaelis-Menten kinetics, with a Kₘ of 4.8 mM and a Vₘₐₓ of 0.125 μmol/h/μg of enzyme (Fig. 7). We have not yet been able to determine the Kₘ and Vₘₐₓ for the enzyme with Glc₃Man₂GlcNAc oligosaccharides because of the limited amounts of substrate available.

As shown in Fig. 7, 2-deoxy-D-glucose markedly enhanced the activity of the enzyme toward pNP-Glc. The substrate activity curve in the presence of 30 mM 2-deoxy-D-glucose follows Michaelis-Menten kinetics. The Vₘₐₓ is increased approximately 3-fold by the activator (compare 0.280 μmol/h/μg to 0.890 μmol/h/μg), but the Kₘ is not greatly affected (1.1 mM versus 0.84 mM). Fig. 8A shows the activation of pNP-Glc activity as a function of the concentration of 2-deoxy-D-glucose and mannose. The former compound was effective at a concentration as low as 12.5 mM. Mannose also enhanced enzymatic activity but to a more modest level and at higher concentrations than 2-deoxy-D-glucose. It seemed possible that the apparent activation might reflect transglucosylation similar to that exhibited by lysosomal acid α-glucosidase (37) or the transgalactosylation shown by β-galactosidase (38). However, neither the expected product of this process, glucosyl-2-deoxyglucose, nor any higher molecular weight product could be detected by thin layer chromatography.

High concentrations of ethylene glycol (70 to 80%), ammonium sulfate (60%, w/v) and α-methylmannoside (1 mM) also activated the enzyme slightly (data not shown), but 2-deoxy-D-galactose (Fig. 8A), N-acetylgalactosamine (50 to 100 mM), and 2-fluoro-2-deoxyglucose (5 to 25 mM) were without effect. Fig. 8B shows the action of various inhibitors on the pNP-glucosidase activity of the enzyme. The most potent inhibitor was p-chloromercuribenzenesulfonate, which gave complete inhibition at 0.8 mM and 50% inhibition at 0.55 mM. The alternative substrate, maltose, served as an effective inhibitor, exhibiting 50% inhibition at 1.8 to 2.0 mM concentration. Inhibition by glucose (50% at 17 mM) is interesting in light of the enhancement of pNP-glucosidase activity by both mannose and 2-deoxy-D-glucose. As expected for a glucosidase, D-glucono-1,5-lactone caused a 50% inhibition at 40 mM. Tris-
The universality of the multistep excision of glucose from nascent glycoprotein biosynthesis in yeast cells (15), CHO cells (6), NIL fibroblasts (4, 5), chicken oviduct (11), bovine thyroid gland (17), calf pancreas (40), calf liver (10), and rat liver (7, 8, 12-14). Although pulse (6) and pulse-chase (6) radiolabeling experiments indicated the three glucose residues were removed in three steps, experiments with partially purified enzymes have indicated that there are only two glucosidases involved (7, 10, 13). For example, during the course of the present work, Ugalde et al. (14) demonstrated with an electrophoresed form of rat liver glucosidase II activity that this preparation cleaves both mono- and diglucosylated oligosaccharides.

The present report describes a procedure for isolating rat liver glucosidase II in highly purified form, permitting further study of its substrate specificity and response to effectors, and the determination of its molecular weight, subunit composition, and other properties. The availability of the purified enzyme should facilitate future studies of its structure and function. Glucosidase II is a neutral α-glucosidase that excises both of the 1,3-linked glucose residues of precursor oligosaccharides after the terminal 1,2-linked glucose has been removed. Since the ratio of the rate of glucose released from precursor oligosaccharides to the rate of glucose released from pNP-α-glucoside remained constant throughout the purification procedure, and since the recovery at each step is reasonably good, it is very likely that we have isolated the major glucosidase II from rat liver microsomes. That this enzyme may be involved in regulating the levels of lipid-linked glucose-containing oligosaccharides was first suggested by the work of Spiro et al. (17) on bovine thyroid gland. This possibility appears to be supported by the recent studies of Cacan et al. (41) and Hoffack et al. (42), who found in testing rat spleen lymphocytes that deglucosylated lipid-linked precursor oligosaccharides were preferentially estabalisaled through the action of a phosphodiesterase to form a free phosphohioginosaccharide. In regard to the roles of the glucose residues in precursor oligosaccharides, a recent report (43) on the biosynthesis of human chorionic gonadotrophic hormone in cultured human choriocarcinoma (JAR) cells may also be mentioned. Only the monoglucosyl derivative could be detected in precursors of the α-subunit of the hormone. It would be of interest to determine whether this observation is a result of the occurrence of a biosynthetic pathway that does not involve di- and triglucosylated intermediates, or, alternatively, an unusually rapid intracellular conversion of these intermediates to the monoglucosyl derivatives.

The fact that a purified α-glucosidase efficiently releases both of the inner 1,3-linked glucose verifies previous findings with crude enzyme preparations (7, 8, 10, 13) and provides further evidence that these residues are linked in the α-configuration. Spiro et al. (17) reported that the chromium trioxide analysis of the calf thyroid precursor oligosaccharide indicated that the glucose residues were α-linked.

Glucosidase II hydrolyzes Glc\textsubscript{Man\textsubscript{2}}GlcNAc at a higher rate than it hydrolyzes Glc\textsubscript{Man}GlcNAc. This finding is consistent with data obtained using partially purified enzyme (7, 10, 13, 14); the Glc\textsubscript{Man}GlcNAc activity (expressed as a percentage of labeled glucose released per unit of time) generally appeared to be 1.5- to 2-fold higher than the Glc\textsubscript{Man\textsubscript{2}}GlcNAc activity. It is difficult to compare the concentrations of the Glc\textsubscript{Man}GlcNAc and Glc\textsubscript{Man\textsubscript{2}}GlcNAc substrates used and the actual rate of glucose release. However, assuming a uniform labeling of the glucose residues and noting that the concentration of Glc\textsubscript{Man\textsubscript{2}}GlcNAc in terms of terminal glucose is only half that of Glc\textsubscript{Man}GlcNAc, then it may be concluded from our studies (Table II) that Glc\textsubscript{Man\textsubscript{2}}GlcNAc is hydrolyzed several times faster than Glc\textsubscript{Man}GlcNAc. This conclusion is in harmony with the results of in vivo pulse and
Pulse-chase radiolabeling studies with chicken embryo fibroblasts (5, 6), which have shown that the first and second glucose residues are removed rapidly, while the last glucose is removed substantially more slowly. (Although compartmentalization may contribute to this observation, it very likely also reflects the substrate preference of glucosidase II.) The tentative conclusion about the relative substrate activity of Glc3Man3GlcNAc and Glc3Man2GlcNAc would not be valid if the two glucose residues were cleaved from the former compound in a concerted sequential manner without the monoglucosyl derivative leaving the active site of the enzyme. Whether or not this occurs cannot as yet be answered.

It should also be mentioned that the yields of glucose release from Glc3Man3GlcNAc never exceeded 80%, although recoveries of added labeled free glucose in these assays were nearly quantitative. Previous investigators (7, 8, 10, 12-14) also generally obtained less than quantitative release of potentially susceptible glucose residues. Whether these results reflect the presence of minor amounts of a different isomer of the oligosaccharide substrate with internal glucosyl residues or the conversion of radiolabeled glucose into mannose during the in vitro merosomal labeling assay (7) is unknown at the present time.

The action of glucosidase II on pNP-α-glucoside or Glc3Man3GlcNAc was not affected by 30 mM turanose, an effective inhibitor of the lysosomal α-glucosidase at 1 to 5 mM (20), nor was it affected by 25 mM kojibiose, an inhibitor of glucosidase I (14).

The effects observed with maltose, glucose, p-chloromercuribenzenesulfonate, and Tris were very similar to those reported for the rat liver glucosidase II activity investigated by Ugalde et al. (13, 14). Similar inhibition of rat liver glucosidase II activity by glucose was also reported by Grinna and Robbins (7). The observations that the highly purified rat liver glucosidase II was not activated by 500 mM phosphate, pH 7.2, and was only slightly activated by 60% (w/v) ammonium sulfate are in contradistinction to the results obtained with the thyroid glucosidase (17). Glucosidase II was not influenced by Na+ or K+, in marked contrast to lysosomal acid α-glucosidase, which is greatly stimulated by mono- and divalent metal ions (27).

Mannose and 2-deoxyglucose activate the pNP-glucosidase activity of glucosidase II. On the other hand, these two sugars inhibit, rather than activate, glucosidase II when the precursor oligosaccharides are used as substrates. Mannose had previously been reported to inhibit the cleavage of Glc3Man3GlcNAc and Glc3Man2GlcNAc (7). A likely explanation for the contrary effects of the two sugars on the hydrolysis of pNP-glucoside, on one hand, and oligosaccharides on the other, is that the two sugars are recognized by a site on the enzyme that normally binds the α-1,2-linked mannosyl residues of a branch of the oligosaccharide adjacent to the one containing glucose. Grinna and Robbins (7, 8) have reported that the glucosidase II activity was three to four times higher with Glc3Man3GlcNAc as substrate than with Glc3Man2GlcNAc, which lacks the two terminal mannoses of the middle branch. Moreover, they report that the cleavage of Glc3Man3GlcNAc was inhibited 80% by the addition of 1 mM Man2GlcNAc to the incubation mixture, a result also suggesting recognition of the α-1,2-linked mannosyl branch (8). In addition, Michael and Kornfeld (10) found that the cleavage of Glc3Man3GlcNAc was many-fold slower than the cleavage of Glc3Man2GlcNAc. Spiro et al. (17) have reported similar results.

While the differing effects of mannose and 2-deoxyglucose on the cleavage of the artificial and oligosaccharide substrates and the slightly different pH optima observed with different substrates (Fig. 6A) might be considered suggestive of the presence of more than one glucosidase in our preparation, the evidence from both our studies and those of others rather strongly suggests that only one enzyme is involved.

Isoelectrofocusing of purified glucosidase II in either sucrose density gradients or polyacrylamide gels showed an apparent PI of 3.5-3.8 for freshly prepared glucosidase II; only one major form was apparent. In our experience, a PI of 3.5-3.8 is low for microsomal proteins, most of which exhibit PI values between 6 and 8 by isoelectrofocusing. This PI is higher than that obtained with freshly prepared Triton extract of washed microsomes (pI = 3.2). These results differ slightly from those recently published by Ugalde et al. (14) in which glucosidase II was isoelectrofocused after elution from a column of concanavalin A-Sepharose. These investigators reported one major form with a pI of 4.2. The fact that the pI increases when glucosidase II is stored prior to further purification suggests the presence of a labile acidic group or the modification of the glycoprotein by some other component of the preparation. The fact that a substantial increase in pI was produced by incubation of the Triton extract with neuraminidases or with endonuclease D, but not with endonuclease H, suggests that oligosaccharide of the complex type may be responsible to some extent for the low pI of glucosidase II. It is possible that neuraminidase present in crude glucosidase II preparations may be the cause of the increase in pI when the preparations are stored.

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