Biochemistry

 Volume 17, Number 3 February 7, 1978

Purification and Properties of β-Glucuronidase from Human Placenta†

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ABSTRACT: β-Glucuronidase from human placenta was purified 18 000-fold to homogeneity in three steps: (1) batch immunoadsorption on antibody–Sepharose resin; (2) DEAE-Sephadex chromatography; and (3) reduction and alkylation followed by DEAE-Sephadex chromatography. The product behaved as a single species when tested by (a) polyacrylamide gel electrophoresis, (b) gel filtration on Sephadex G-200, (c) sedimentation equilibrium data, and (d) double immunodiffusion against either goat or rat antibody to β-glucuronidase. The molecular weight determination gave values of 310 000 ± 10 000 (gel filtration) and 286 000 ± 10 000 (sedimentation equilibrium). Amino acid analysis showed no cysteine. No radioactivity was incorporated into the protein upon reduction and alkylation with [^{14}C]iodoacetic acid. The carbohydrate analysis indicated the following number of carbohydrate residues (per 286 000 mol wt): mannose, 68; *N*-acetylglucosamine, 19; galactose, 12; glucose, 13; fucose, 15; sialic acid, 0. Optimal catalytic activity was found at pH 3.8 in the pH range 3.6–5.8. The thermal stability at 70 °C is optimal at pH 5.85. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate revealed three subunits, with the molecular weights of 77 000, 60 000, and 18 000. This result, with recent genetic evidence (Chern, C. J., and Croce, C. M. (1976), *Am. J. Hum. Genet. 28,* 350) for a single structural gene for β-glucuronidase, is compatible with the view that human placenta β-glucuronidase is a tetramer of 77 000-dalton subunits some of which may consist of 60 000- and 18 000-dalton polypeptides.

β-Glucuronidase (EC 3.2.1.31) is a lysosomal acid hydrolase which has been studied extensively in different mammalian systems. Rat liver β-glucuronidase (de Duve et al., 1955) and mouse liver β-glucuronidase (Paigen, 1961; Fishman et al., 1969) are found in both lysosomes and microsomes. Mouse liver β-glucuronidase has multiple forms (Swank and Paigen, 1973), tetrameric structure, carbohydrate residues, and only trace amounts of cysteine (Tomino et al., 1975). The β-glucuronidase from rat liver (Stahl and Touster, 1971; Owens et al., 1975) or rat preputial gland (Himeno et al., 1974; Tulsiani et al., 1975) has chemical, physical, and biochemical properties similar to the mouse enzyme. β-Glucuronidase from bovine liver (Plapp and Cole, 1966) was found to have multiple forms which differed in carbohydrate content and isoelectric point (Plapp and Cole, 1967). Human placental enzyme has been partially purified (Contractor and Shane, 1972; Contractor and Oakey, 1977) and the human liver enzyme purified to apparent homogeneity (Musa et al., 1965). However, the properties of the latter differ from those we report here, possibly because of harsh conditions during the initial purification step (an overnight autolysis at 37 °C).

In man, the normal role of β-glucuronidase in the lysosomal degradation of mucopolysaccharides was revealed by the discovery of a storage disease (Sly et al., 1973) which was associated with the enzyme deficiency (Hall et al., 1973). In vitro (fibroblast) (Brot et al., 1974) and in vivo (animal) (Achord et al., 1977) models have been developed to study the potential role of enzyme replacement therapy in this disease. The need for pure enzyme in these studies of experimental enzyme replacement stimulated us to purify and characterize β-glucuronidase from human placenta.

Experimental Procedures

Materials

Urea, human IgG,[1] ovalbumin, and myoglobin were obtained from Schwarz/Mann; sodium deoxycholate and sodium dodecyl sulfate were from Fisher; Naphthol ASBI-β-D-glucuronide, naphthol blue black, pararosaniline hexazonium salt, Coomassie Blue G-250, dithiothreitol, *p*-nitrophenyl β-D-glucuronide, bovine serum albumin, Tris, yeast alcohol dehydrogenase, and iodoacetic acid were obtained from Sigma; the Pharmacia products Sepharose 2B, DEAE-Sephadex, Blue

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; IgG, immunoglobulin G; ME medium, minimum Eagle's medium; ASBI, 6-bromo-2-hydroxy-3-naphthoyl-*o*-anisidide.

Dextran, and Sephadex G-200 were obtained from Sigma; cyanogen bromide, *N,N,N′,N′*-tetramethylethylenediamine, *N,N′*-methylenebisacrylamide, acrylamide, and thiobarbituric acid were from Eastman; *E. coli* β-galactosidase was from Boehringer Mannheim; 4-methylumbelliferyl β-D-glucuronide and 4-methylumbelliferone were from Research Products International; goat antisera to rat preputial gland β-glucuronidase was purchased from Bio Tek, Inc. Goat anti-human IgG (γ-heavy chain specific) was obtained from Meloy (Springfield, Va.), and rabbit anti-goat IgG (heavy and light chain specific) was prepared in our own laboratory.

For gas chromatographic analyses, methanolic hydrogen chloride was from Supelco, 3% SE-30 on Chromosorb Q was from Applied Science Labs, and analytical standard sugars were from Pfanstiehl Co. All other reagents were of analytical grade or the best grade available.

Methods

Preparation of Antibody–Sepharose. Goats were immunized with rat preputial gland β-glucuronidase (Tulsiani et al., 1975). IgG was obtained from the resulting antisera by ammonium sulfate precipitation (0–40%) and DEAE-cellulose chromatography at pH 7.0 in 0.02 M phosphate buffer (Heide and Schwick, 1973; Johnson and Holbarrow, 1973). The protein was made 0.2 M in citrate, pH 6.5, and frozen or bound to agarose. Purified IgG was coupled to cyanogen bromide activated Sepharose 2B (0.3 g of cyanogen bromide/mL of settled Sepharose) in a ratio of 0.9–1.25 g of IgG/100 mL of settled Sepharose (Brot et al., 1974) by the general method of Cuatrecasas and Anfinsen (1971).

Human placentas were obtained from the Barnes Hospital, St. Louis, Mo., or the Jewish Hospital of St. Louis. They were refrigerated immediately after delivery, then washed, cut up, and frozen within 24 h.

Purification of β-Glucuronidase: 1. Tissue Extraction and Batch Affinity Chromatography. Frozen placenta tissue (1.2 kg) was homogenized in a commercial Waring blender in 3.6 L of buffer (0.1 M sodium chloride, 0.05 M Tris-HCl, pH 8.0). The mixture was centrifuged 20 min at 1740*g* and the supernatant recentrifuged at 2500*g*. The final supernatant solution was filtered through cheesecloth and adsorbed batchwise on 150–200 mL of antibody–Sepharose for 8 h at 4 °C on a rotary shaker. The resin was then filtered and sequentially washed with 3 volumes each of cold solutions: 0.15 M sodium chloride, 1 M sodium chloride, distilled water. β-Glucuronidase was eluted from the resin by 6 M urea.

2. First DEAE-Sephadex Chromatography. The urea extracts containing 30–35 × 10^6 units (accumulated from two to three repetitions of the first step) were made 3 M in urea and 0.01 M in Tris-HCl, pH 8.0, and adsorbed to a DEAE-Sephadex (A-25) column (2.5 × 33 cm) equilibrated in the same buffer. After the charged resin was washed with three volumes of 0.01 M Tris-HCl, pH 8.0, the enzyme was eluted with 850 mL of a sodium chloride gradient (0–0.3 M) in the same buffer. A fivefold concentration by dialysis at reduced pressure or by ultrafiltration (in an Amicon Model 52 with an XM-50 membrane) produced an enzyme preparation 5640-fold purified from the crude extract. This preparation contained an impurity identified as human IgG (see below).

3. Reduction and Alkylation, and Second DEAE-Sephadex Chromatography. Concentrated β-glucuronidase from the above steps, 12 × 10^6 units (1 mg/mL), was incubated with 0.01 M dithiothreitol in 6 M urea, 0.1 M Tris-HCl, 0.025 M NaCl for 1–2 h at 37 °C under nitrogen atmosphere, and then treated with iodoacetic acid (0.03 M, 1.5 equiv) for 30 min. Under these conditions, cysteine residues were alkylated completely (Gall et al., 1968). The total volume was 35 mL. The mixture was then dialyzed four times against 3 L of 10 mM Tris, pH. 8.0.

The dialyzed mixture was applied to a 1.5 × 15 cm column of DEAE-Sephadex, equilibrated in 10 mM Tris-HCl, pH 8.0. The resin was washed with the same buffer and eluted with a sodium chloride gradient (0–0.3 M) in that buffer. Peak fractions were combined and concentrated with a Millipore microfiltrator to a concentration of 0.25 mg/mL (1.0 × 10^6 units/mL).

Determination of Cysteine by Radioactive Labeling. β-Glucuronidase from the first DEAE-Sephadex chromatography was reduced as described above and alkylated first with 0.7 mg of [1-^{14}C]iodoacetic acid (13.8 mCi/mmol) for 30 min at room temperature in the dark and then with a 50% molar excess of iodoacetic acid for another 30 min. The alkylated mixture was then treated as above. The radioactivity was measured by adding 0.1 mL of each fraction to 0.9 mL of water and 10 mL of RPI 3a-70 scintillation cocktail.

Enzymatic Assay. Enzymatic activity was determined fluorometrically using 10 mM 4-methylumbelliferyl β-D-glucuronide in 0.2 M sodium acetate, pH 4.8, as described (Brot et al., 1974). One unit of enzyme activity hydrolyzes 1 nmol of substrate per h under assay conditions. The fluorescence is measured in a Farrand ratio fluorometer using a Corning No. 7-60 and Kodak Wratten ND as primary filters and Kodak Wratten No. 2A and No. 48 as secondary filters. Human β-glucuronidase was assayed at pH 4.8 except for measurements of the pH–rate profile (Figure 4).

Protein Determination. The method of Lowry et al. (1951) was used with bovine serum albumin as standard.

Analytical Polyacrylamide Gel Electrophoresis. The polyacrylamide gels (5%) were prepared in tubes at pH 9.5 according to the method of Davis (1964). Enzyme activity was demonstrated with the histochemical stain, Naphthol-ASBI-glucuronic acid, according to the procedure of Stahl and Touster (1971). The mobility of human enzyme in the pH 8.1 buffer system was insufficient for analysis, although mobility of the mouse enzyme in this system was sufficient to detect multiple forms (Tulsiani et al., 1975).

Polyacrylamide gels with sodium dedecyl sulfate were prepared in 5-mm tubes (10% gel) in Tris–glycine buffer as described by Laemmli (1970). The protein bands were visualized with 0.1% Coomassie Blue G-250 as described by Weber and Osborn (1969) and destained overnight in 7% acetic acid at 50 °C.

Preparation of Antisera and Immunological Determination of Enzyme Purity. The purity of the human placenta β-glucuronidase was assessed by injecting the enzyme into animals and assaying the resulting antisera for antibody activity to other serum and tissue proteins in addition to β-glucuronidase. A goat was immunized by injection of 100 μg of protein emulsified in complete Freund's adjuvant. Thirty days later 100 μg of protein emulsified in incomplete Freund's adjuvant was injected subcutaneously, and the animal was bled 7 days later. Each of eight rats was immunized by intramuscular injection of 50 μg of protein emulsified in complete Freund's adjuvant. The animals were bled 30 days later and the sera pooled. Antisera were assayed against various antigens by double immunodiffusion in 0.6% agarose gels in 0.04 M phosphate, 0.15 M sodium chloride, pH 7.2. Gels were washed in the same buffer, then in deionized water, dried, and stained for protein with 0.45% naphthol blue black. Other gels were stained for β-glucuronidase activity by the simultaneous diazo coupling method of Hayashi et al. (1964). The presence of human and goat IgG in β-glucuronidase preparations was

TABLE I: Purification of Placenta β-Glucuronidase.

Steps	Total protein (mg)	Total[a] act. (units)	Spec act. (units/mg)	Recovery (%)	Purification
Crude extract	259 000	58.3×10^6	225	100	1
Antibody-Sepharose	432	35×10^6	81 000	60	360
DEAE-Sephadex no. 1	11	14×10^6	1.27×10^6	24	5 640
DEAE-Sephadex no. 2[b]	1.7	7×10^6	4.08×10^6	12	18 130

[a] Units are the number of nanomoles of 4-methylumbelliferone released/h from 10 mM 4-methylumbelliferyl β-D-glucuronide at pH 4.8. [b] Prior to this step, the preparation is treated with dithiothreitol and iodoacetic acid as described in Methods.

measured by immunodiffusion against goat anti-human IgG (γ-heavy chain specific) and rabbit anti-goat IgG (heavy and light chain specific).

Molecular Weight by Gel Filtration. Analytical gel filtration on Sephadex G-200 (1.6 × 100 cm) was performed by ascending chromatography at a flow rate of 8 mL/h. The column was preequilibrated with 0.075 M sodium chloride, 0.02% sodium azide, 0.01 M Tris-HCl, pH 8.0. The enzyme sample (3 mL) contained the protein markers. The void volume was determined with Blue Dextran 2000, β-galactosidase was determined by the method of Kennell and Magasanik (1964), and β-glucuronidase by the above method. The elution volume of the marker proteins was determined by the absorbance at 280 nm or enzymatic assay of the collected fractions (1 mL). The molecular weight of β-glucuronidase was calculated from a plot of the log molecular weight of the standard proteins vs. the observed elution volume of these proteins.

The following marker proteins were used: β-galactosidase from *E. coli* (480 000), yeast alcohol dehydrogenase (158 000), bovine serum albumin (68 000), ovalbumin (46 000), and myoglobin (17 500).

Molecular Weight Determined by Analytical Ultracentrifugation. Sedimentation equilibrium analysis was carried out on a Beckman Model E analytical centrifuge equipped with an AN-H rotor. Samples of the protein previously dialyzed against 0.075 M NaCl, 0.01 M Tris, pH 8.0, 0.02% sodium azide for 4 h at 4 °C were centrifuged at 10 000 rpm for 72 h. Protein distribution at 280 nm was measured with a Beckman photoelectric scanner. To ensure that equilibrium had been attained several scans were taken during the run.

The molecular weight of the protein was calculated from the plot of ln A_{280} vs. X^2 according to the standard method (Chervenka, 1969). The value of the partial specific volume of the protein (0.719 cm^3/g) was calculated from the partial specific volume of the amino acid residues (Cohn and Edsall, 1943) and of the carbohydrate residues (Gibbons, 1972).

Amino Acid Analysis. The amino acid composition of β-glucuronidase was determined using a Beckman Model 120C amino acid analyzer following a 24-h hydrolysis in 6 N HCl as described by Moore and Stein (1963).

Determination of Carbohydrates. The composition was estimated by the phenol and sulfuric acid method (DuBois et al., 1956). Neutral sugars and amino sugars were determined by gas chromatography of the trimethylsilyl ethers after methanolysis of the glycoprotein, and N-acetylation, and de-O-acetylation of the resultant methyl glycosides according to the modification (Baenziger et al., 1974; Kornfeld and Ferris, 1975) of Reinhold's procedure (Reinhold, 1972). Analysis was performed on a 6 ft. × 2 mm glass column (3% SE-30 on Chromosorb Q) in a Varian 1400 gas chromatograph temperature programmed at 2 °C per min from 120 to 260 °C. Arabitol was used for the internal standard. Areas were computed by triangulation. Sialic acid was determined by the method of Warren (1959).

Kinetics. The thermal stability and pH–rate profile were determined as described in the figure legend. The apparent K_m was determined by assaying 1000 units of β-glucuronidase at 37 °C, 30 min in 0.2 M acetate buffer at pH 4.8 with 1, 2, 5, and 10 mM 4-methylumbelliferyl β-D-glucuronide or *p*-nitrophenyl β-D-glucuronide. The K_m values were obtained from a double-reciprocal plot of initial substrate concentration vs. rate of product formed.

Pinocytosis Measurements. Cultured fibroblasts from a patient with β-glucuronidase deficiency mucopolysaccharidosis were grown as previously described (Kaplan et al., 1977a) in Eagle's ME medium with Earle's salts (GIBCO) supplemented with 15% heat-inactivated fetal calf serum and 3 mM glutamine. To determine the amount of β-glucuronidase taken up by the cell cultures, 35-mm Petri dishes containing cells at confluence (approximately 0.2 mg of protein per plate) were exposed to 7000–10 000 units of enzyme in 1 mL of this medium. After a 24-h incubation at 37 °C, dishes were chilled and washed six times with 0.02 M phosphate, 0.15 M sodium chloride, pH 7.2, as previously described (Kaplan et al., 1977a). These washes were sufficient to remove all absorbed enzyme. The cells were then lysed with 0.5 mL of 1% sodium deoxycholate. The lysates were assayed for β-glucuronidase activity and for cell protein. The results are expressed as uptake ratios: cell associated activity divided by the total activity supplied in the medium.

Gel Scanning and Molecular Weight of Subunits. Optical density of the stained sodium dodecyl sulfate–polyacrylamide gels was determined at 600 nm in a Gilford 2400 recording spectrophotometer equipped with a Gilford 2410 gel scanner. The molecular weight of subunits was determined from a plot of log molecular weight vs. mobility of marker proteins: bovine serum albumin (68 000), reduced IgG (50 000 for heavy chain, 25 000 for light chain), and myoglobin (17 500).

Results

Purification. Purification of β-glucuronidase from human placenta was achieved (see Table I) in three steps by use of affinity chromatography on a heterologous immunoadsorbent resin, ion-exchange chromatography on DEAE-Sephadex resin, and reduction and alkylation, followed by repeat ion-exchange chromatography. Batchwise adsorption of crude extract to immunoadsorbent resin and desorption with 6 M urea provided enzyme in 60% yield with a 360-fold increase in specific activity. The resin can adsorb at least 90% of the activity when previously unused. When adsorption of crude activity fell below 80%, the resin was washed exhaustively with 4 M potassium thiocyanate. Antibody resin maintained in this way was good for about 40 isolations.

The ion-exchange chromatography of the pooled urea extracts on DEAE-Sephadex (Figure 1) showed heterogeneity in catalytic activity as well as the uptake ratio of the β-glucuronidase. Although the uptake ratios of β-glucuronidase fractions from placenta enzyme did not approach the uptake

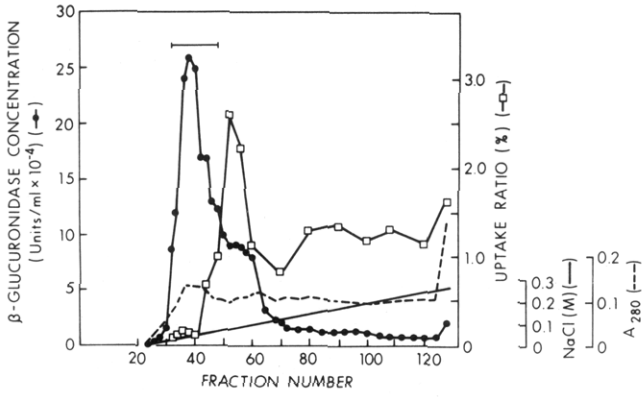

FIGURE 1: DEAE-Sephadex chromatography of partially purified human placenta β-glucuronidase at pH 8 in 0.01 M Tris-HCl with sodium chloride gradient 0–0.3 M. The fractions (5 mL) were assayed for β-glucuronidase activity and protein (A_{280}). The uptake ratio was determined in 35-mm plastic dishes as described in Methods. Fractions under the horizontal line were pooled for further purification.

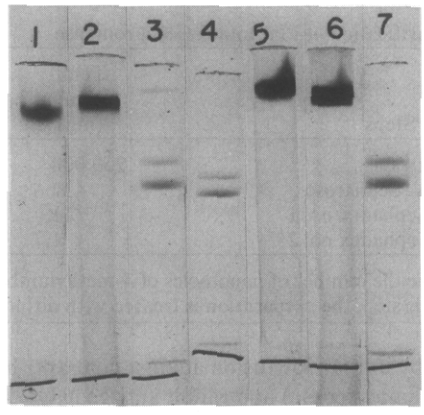

FIGURE 2: Polyacrylamide gels of β-glucuronidase from the first DEAE-Sephadex chromatography stained for protein (no. 1, 16 μg) or activity (no. 2, 0.2 μg) and from the second DEAE-Sephadex chromatography stained for protein (no. 5, 25 μg) or activity (no. 6, 0.2 μg). Sodium dodecyl sulfate–polyacrylamide gels of β-glucuronidase from the first DEAE-Sephadex chromatography without mercaptoethanol (no. 3, 33 μg), with mercaptoethanol (no. 4, 33 μg), and from the second DEAE-Sephadex chromatography without mercaptoethanol (no. 7, 33 μg). A wire marks the position of the tracking dye, bromophenol blue.

ratios reported for high-uptake enriched human platelet enzyme fractions (Kaplan et al., 1977a), the placenta enzyme with relative greater uptake ratio eluted above 0.08 M NaCl as was the case with platelet enzyme. Pooled high uptake activity (fractions 49–60) on polyacrylamide gel electrophoresis showed one band which stained for β-glucuronidase activity. The mobility of this band was slightly faster than that of low uptake activity (Figure 2, gel 2).

Pooled activity (fractions 32–48), which eluted at 0.035–0.075 M NaCl, was obtained in 40% yield with a 15-fold increase in specific activity. Polyacrylamide gel electrophoresis of this enzyme showed one band in the Davis system which stained for β-glucuronidase activity as well as protein (Figure 2, gels 1 and 2). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970) of the same preparation revealed a pattern of multiple bands which was modified by pretreatment (gel 4) with mercaptoethanol. Upon reductive treatment (gel 4) the slowest moving of four bands (high molecular weight) disappeared. Instead, two faster moving bands (lower molecular weight), only one of which is obvious in the photograph, now appeared. The other three bands stayed in the same position (Figure 2, gel 3 and gel 4).

IgG was suspected as an impurity in the β-glucuronidase preparation after the first DEAE-Sephadex chromatography because the relative mobilities of the reduced bands corresponded to those of the subunits of authentic human IgG. The remaining three bands, whose position was unaffected by mercaptoethanol treatment, were tentatively assigned to β-glucuronidase. The presence of human IgG in the enzyme preparation was confirmed by double immunodiffusion with goat anti-human IgG (γ-heavy chain specific) antiserum. No precipitin line was obtained with rabbit anti-goat-IgG antiserum. Chromatography on concanavalin A-Sepharose, aminohexyl-Sepharose, Protein A–Sepharose, and Sepharose coupled to goat anti-human IgG was ineffective in removing the IgG from β-glucuronidase.

β-Glucuronidase from two mammalian sources has been found to contain no cysteine residues (Himeno et al., 1974, 1975). We reasoned that, if the same were true for human β-glucuronidase, we might exploit this fact to separate the enzyme from IgG which contains both interchain and intrachain disulfide bonds (Edelman et al., 1968). Accordingly the products of the first DEAE-Sephadex step containing both proteins were subjected to reductive alkylation with the result that the IgG contaminants were removed by the second DEAE chromatography. Under the conditions used to alkylate IgG, no carboxymethyl group was incorporated into β-glucuronidase.

Criteria for Homogeneity. The homogeneity of β-glucuronidase from step 4 of Table I was demonstrated by several methods. First, polyacrylamide gel electrophoresis of this preparation in the Davis system showed one band which stained for enzyme activity and for protein (see gels 5 and 6, Figure 2). However, gel electrophoresis in the presence of sodium dodecyl sulfate showed the same three subunits (found in the earlier preparation of β-glucuronidase) that did not change if the sample was treated with mercaptoethanol prior to electrophoresis.

Second, gel filtration on Sephadex G-200 gave a symmetrical peak of enzyme activity. Third, sedimentation equilibrium determinations gave a linear plot of ln A_{280} vs. X^2. These are discussed below under molecular weight determinations. Finally, the analysis of immune goat and rat antisera by double immunodiffusion gels (Figure 3) showed a single precipitin line of complete identity with the immunizing enzyme and the crude extract. No precipitin lines were formed with normal human serum or normal goat serum. Analysis of further dilutions of either antigen or antisera failed to reveal additional precipitin lines. When the gels were stained for β-glucuronidase activity, only the single precipitin line stained. Goat antisera to the enzyme that was purified through the first DEAE-Sephadex chromatographic step showed two precipitin lines with the enzyme preparation: one antigen was identified as β-glucuronidase (Bell et al., 1977); the other has subsequently been identified as human IgG (unpublished observation). An effort was made to detect human and goat IgG in the enzyme purified through the final DEAE-Sephadex step. Double immunodiffusion of the enzyme preparation of 100 μg/mL against serial twofold dilutions of goat anti-human IgG and rabbit anti-goat IgG failed to develop any precipitin lines. Thus, neither human nor goat IgG could be detected in the enzyme preparation by these methods. Under the conditions of our assay, a 1% or greater contamination by IgG could have been detected.

Molecular Weight Determinations. As described earlier gel filtration of placenta β-glucuronidase on Sephadex G-200 gave

TABLE II: Amino Acid Analysis of β-Glucuronidase from Human Placenta.

Amino acid	μmol/mg[a]	Residues/mol[b]
Lys	0.273	78.0
His	0.169	48.3
Ammonia	1.031	
Arg	0.350	100.1
Asp	0.48	137.3
Thr	0.296	84.7
Ser	0.295	84.4
Glu	0.55	157.3
Pro	0.323	92.4
Gly	0.356	101.8
Ala	0.300	85.8
½-cystine[c]	0	0
Val	0.333	95.2
Met	0.068	19.4
Ile	0.177	50.6
Leu	0.495	141.6
Tyr	0.271	77.5
Phe	0.219	62.6

[a] Determined by 24-h hydrolysis in 6 N HCl. [b] Expressed as the number of residues/mol of protein using molecular weight of 286 000. [c] Determined as CM-cysteine.

TABLE III: Carbohydrate Analysis of Placental β-Glucuronidase.[a]

Residue	mg/100 mg of protein	Moles/mole of protein[b]
Man	4.25	67.5
Fuc	0.87	15.2
Gal	0.77	12.2
Glc	0.80	12.7
GlcNAc	1.46	18.9
GalNAc[c]	<0.15	0
Sialic acid[d]	<0.08	0

[a] The average of three determinations. Standard deviation was 10–20%. [b] Expressed as the number of each residue using the value of 286 000. [c] Not detectable. Under conditions of gas chromatography, the main peak for GalNAc appears with a satellite peak of GlcNAc. Therefore this is an uncertain value due to high background. [d] Determined by the method of Warren (1959). One nanomole of sialic acid per nmol of protein could have been detected by this method.

a symmetrical peak. The elution volume of this peak corresponded to a molecular weight of 310 000 ± 10 000 from the plot of log molecular weight vs. elution volume of the indicated standard proteins. In addition, the β-glucuronidase from rat preputial gland (Himeno et al., 1975; Keller and Touster, 1975) gave the expected molecular weight (~300 000) on this column.

The molecular weight of β-glucuronidase was further measured by sedimentation equilibrium studies in the Beckman Model E analytical ultracentrifuge. Sedimentation equilibrium of the sample for 72 h at 10 000 rpm gave a linear plot of ln A_{280} vs. X^2. By using the partial specific volume calculated from the amino acid and carbohydrate analyses (see Tables II and III), the molecular weight of β-glucuronidase was calculated to be 286 000 ± 10 000.

Composition. The amino acid composition of placenta β-glucuronidase is given in Table II. The results are expressed as number of residues of each amino acid per whole protein molecule. The notable feature is the confirmation of the absence of cysteine. Lack of cysteine or extremely low values have been found in β-glucuronidase from other sources (see Discussion).

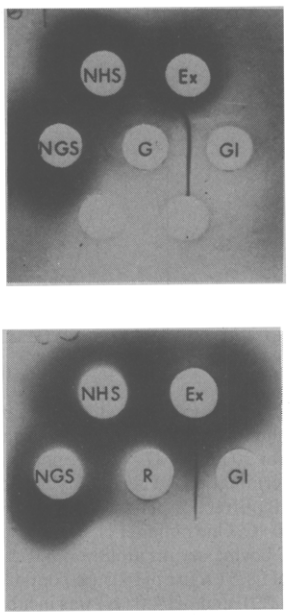

FIGURE 3: Double immunodiffusion gels of goat or rat anti-human placenta β-glucuronidase with β-glucuronidase preparations and normal sera. Upper template shows *goat* anti-human placenta β-glucuronidase (G) in center well and, clockwise in outer wells, normal goat serum undiluted (NGS), normal human serum undiluted (NHS), crude extract of human placenta β-glucuronidase (Ex) 70 μg/mL, and β-glucuronidase from step 4 of Table I (GI) 100 μg/mL. Lower template shows *rat* anti-human placenta β-glucuronidase (R) in center well and same samples in the outer wells. Gels were stained for protein.

The carbohydrate content was estimated as 6.5% by the phenol–sulfuric acid test (DuBois, 1956) using mannose as standard. A carbohydrate content of 8.15 mg/100 mg of protein can be calculated from data given in Table III. The results are also expressed as moles of sugar/mole of whole protein. The preponderance of mannose (67.5 residues) is consistent with the high affinity of this protein for concanavalin A. N-Acetylglucosamine is the next highest occurring monosaccharide (19 residues). Galactose, fucose, and glucose appear equally, although the origin of glucose from nondialyzable dextrins from the Sephadex resins has not been excluded. Glucose was proven to be a constituent of rat preputial gland β-glucuronidase (Tulsiani et al., 1975). Unfortunately, lack of pure material prevented analysis of human β-glucuronidase by those methods. Neither sialic acid nor galactosamine was found.

Kinetics. The optimal pH for activity is 3.8. At this pH the catalytic activity is 1.8 times the value calculated at pH 4.8, the usual assay pH (Figure 4). From the pH of optimal activity a gradual decrease in activity with increasing pH was found. β-Glucuronidase was assayed at pH 4.8 in acetate buffer to allow for comparison of enzyme activity from other sources, for use with other substrates, and for use with enzymes at any degree of purity. At pH 4.8 the K_m for placenta β-glucuronidase with 4-methylumbelliferyl-β-D-glucuronide was 1.30 ± 0.10 mM and that with *p*-nitrophenyl β-D-glucuronide was 2.90 ± 0.30 mM.

Thermal stability at various pHs was determined in the presence of 1 mg/mL bovine serum albumin and either 0.02 M Tris-HCl or 0.02 M acetate as indicated in Figure 4. Thermal stability at 70 °C is optimal at pH 5.85 and decreases as pH is increased or decreased in the range 4.0–8.5. Periodate treatment or acylation of this enzyme (presumably the lysine ε-amino groups) decreases the temperature at which thermal inactivation occurs (at pH 5.85) to 65 °C for the periodate treated and <65 °C for the acylated β-glucuronidase. Catalytic

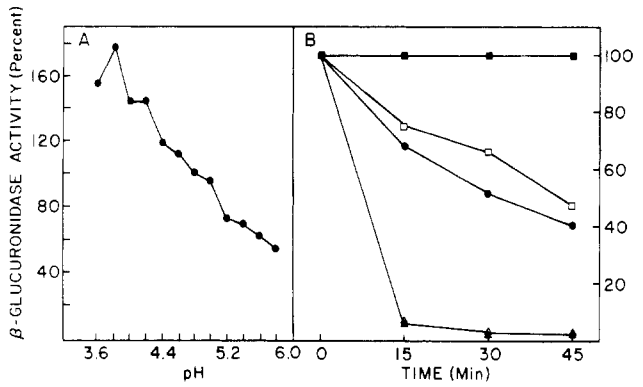

FIGURE 4: (A) Effect of pH on activity. One thousand units were assayed with 10 mM 4-methylumbelliferyl β-D-glucuronide in 0.2 M acetate buffer at the indicated pH for 30 min at 37 °C. The points represent an average of two duplicate determinations. The activity is expressed as percent of activity at pH 4.8. (B) Effect of pH on thermal inactivation of placenta β-glucuronidase at 70 °C. One milliliter of a solution containing 1000 units of enzyme, 1 mg of bovine serum albumin, 0.075 M NaCl in 0.02 M Tris-HCl buffer at pH 8.5 (▲) or pH 7.5 (●) or 0.02 M acetate buffer at pH 5.85 (■), pH 4.8 (□), and pH 4.0 (△) was incubated for the indicated time intervals. After cooling in ice-water, the samples were assayed 30 min with 4-methylumbelliferyl β-D-glucuronide in 0.2 M acetate at pH 4.8, 37 °C. The enzyme activity in samples incubated at 0 °C was not affected (less than 5%).

activity at 37 °C and pH 4.8 is unaffected by these treatments of the enzyme.

Subunit Analysis. The treatment of placenta β-glucuronidase with 0.1% sodium dodecyl sulfate for 2 h at 37 °C causes the complete dissociation of the protein into subunits as determined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. The molecular weights of these subunits were calculated to be 77 000, 60 000, and 18 000. Calculations from gel scans show that 30% of the protein exists as a 77 000-dalton subunit, and 70% as 60 000- and 18 000-dalton subunits by assuming the area (intensity of stain) is proportional to the molecular weight of the subunit.

Discussion

Of several methods used to determine the homogeneity of the purified β-glucuronidase from human placenta, immunodiffusion provided the strongest evidence that the final preparation is homogeneous (at least 99% pure). The antigenic purity of human β-glucuronidase was determined by analysis of immune serum obtained from two different hosts, goat and rat (Figure 3). Goat serum was chosen since the goat is very sensitive to human proteins. The goat antiserum to human β-glucuronidase should contain antibodies to other human protein in the preparation. Goat IgG, possibly dissociated from the immunoadsorbent resin (Yong, 1973), would not be immunogenic in this system. Analysis of immune rat serum was chosen because the rat can generate antibodies to both human and goat proteins. Since only antibodies to human β-glucuronidase could be detected by double diffusion and immunoelectrophoresis, we judged our protein to be immunologically pure within the limits of these methods.

Each of the other methods supporting the homogeneity of the final enzyme had a greater limitation. From sedimentation equilibrium data the slope of the plot would be unaffected by an impurity of 5% or less if it was of lower molecular weight than β-glucuronidase. In gel filtration experiments, only enzyme activity was measured. Nonenzymatic protein was not monitored because of the large amounts of enzyme protein required for this technique. Although polyacrylamide gel electrophoresis of partially purified β-glucuronidase in the Davis system disclosed no heterogeneity, electrophoresis of that preparation in the presence of sodium dodecyl sulfate revealed human IgG as an impurity. The copurification of human IgG is presumed to occur because its isoelectric point, pI = 7.3–8.1 (Cohn, 1950), overlaps the isoelectric point of β-glucuronidase from human placenta, pI = 7.3–7.5 (Glaser et al., 1975). To separate IgG from the enzyme in this protein mixture advantage was taken of the lack of cysteine in β-glucuronidase. The cysteine-containing IgG (Edelman et al., 1968) contaminant was selectively modified (by alkylation of cysteine) and final purification was achieved by ion-exchange chromatography.

β-Glucuronidase from human placenta was found to be similar to β-glucuronidase from mouse liver (Tomino et al., 1975), from rat preputial gland (Tulsiani et al., 1975; Himeno et al., 1975), from rat liver (Stahl and Touster, 1971) and bovine liver (Himeno et al., 1974) in regard to (a) molecular weight, (b) tetrameric structure and subunit molecular weight (see below), (c) lack or low content of cysteine, and (d) carbohydrate composition that is high in mannose.

Genetic analysis of β-glucuronidase with mouse–human hybrid cells (Chern and Croce, 1976) and with Chinese hamster–human hybrid cells (Franke, 1976) allowed assignment of the structural gene for β-glucuronidase to human chromosome 7. Tetrameric structure composed of identical subunits has been proposed for mouse and rat β-glucuronidase. The hybrid enzymes formed in man–mouse and man–hamster hybrids argue for tetrameric structure for the human enzyme also. We found that, in the presence of sodium dodecyl sulfate, β-glucuronidase dissociates into three different subunits, and that the sum of the molecular weights of the two smaller subunits equals the molecular weight of the largest subunit. If there is only one structural gene for human β-glucuronidase, it is possible that the largest subunit (monomer) is structurally related to the two smaller subunits. They may be generated by a proteolytic cleavage in a major portion of the monomers. Recently a similar phenomenon was documented for fructose 1,6-bisphosphatase obtained from rabbit liver. This tetrameric enzyme was sensitive to proteolytic cleavage by a lysosomal protease (Pontremoli et al., 1973) and by subtilisin (Traniello et al., 1972; Dzugaj et al., 1976). However, conversion of the 36 000-dalton monomer to 29 000- and 6000-dalton fragments became apparent only on sodium dodecyl sulfate gel electrophoresis or chromatography under dissociative conditions (Dzugaj et al., 1976). In addition Himeno et al. (1976) observed that electrophoresis of rat liver lysosomal β-glucuronidase in sodium dodecyl sulfate–polyacrylamide gels shows bands which correspond to three subunits (molecular weights of 79 000, 74 000, and 70 000). It is possible that the same type of fragmentation may be occurring in these examples.

We tentatively interpret the molecular weight value of the holoenzyme, the genetic evidence for a structural gene of human β-glucuronidase located in one chromosome, and the subunit information presented above, in this way: β-glucuronidase is a tetramer of identical subunits of molecular weight 77 000. Some of these single polypeptide chains are cleaved, either in lysosomes or during isolation, into 60 000 and 18 000 molecular weight fragments which remain associated in the tetramer structure and dissociate only in sodium dodecyl sulfate. This interpretation could be tested by a comparison of the terminal amino acid sequences of the smaller subunits with those of the largest subunit.

The carbohydrate structure of human β-glucuronidase has recently been found to be important for recognition of this enzyme by cultured human fibroblasts (Kaplan et al., 1977a) and for clearance from rat plasma following infusion (Achord

et al., 1977). Forms of placenta β-glucuronidase which show high-uptake in fibroblasts (a minority component ~20%) appear to have a phosphomannose component that is recognized by fibroblast pinocytosis receptors. Treatment of this high-uptake enzyme with alkaline phosphatase abolishes its recognition and uptake by fibroblasts (Kaplan et al., 1977b). The low-uptake form for fibroblasts (the predominant component of enzyme from placenta) is cleared from rat plasma by a mannose recognition system. Periodate treatment of the enzyme converts the placenta enzyme from a rapid to a slow clearance form.

Acknowledgments

We are indebted to Barnes Maternity Hospital and the Jewish Hospital of St. Louis for cooperation in supplying placentas. We thank the following persons for capable technical assistance: Mr. Richard Hiller, Mr. Roger Urbani, Ms. Sarah Sutherlin, and Ms. Kathleen Georgen. We thank Professor E. Simms and Dr. S. Bridges for advice on immunoglobulins, Dr. W. Nulty for running the ultracentrifuge and helping with data interpretation, Dr. R. Kornfeld for excellent advice on determination of carbohydrates by gas chromatography, Dr. R. Bradshaw and Mr. O. Bates for amino acid analysis, and Drs. D. Achord and A. Kaplan for valuable advice.

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