The Preparation and Chemical Composition of the Multiple Forms of β-Glucuronidase from the Female Rat Preputial Gland*

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β-Glucuronidase isolated from the preputial gland of the female rat has previously been shown to be a tetrameric glycoprotein. We have now separated the enzyme into several molecular forms by chromatography on hydroxylapatite columns. The three major forms (A, B, and C) have a very similar or identical amino acid composition, and kinetic and stability studies on forms B and C disclosed no differences between these two forms. However, form C contained much more carbohydrate than forms A and B, which were very similar in carbohydrate composition. The sugars in forms A and B are mannose (2.8%), glucosamine (1.9%), fucose (0.2%), galactose (0.16%), and glucose (0.17%). Form C is a little higher in mannose content, but, more distinctively, is much richer in fucose (0.6%), galactose (1.1%), and glucose (1.5%). The presence of glucose was established by paper chromatography as well as by gas-liquid chromatography, and several special experiments were performed to rule out the possibility that this hexose was present in a persistent contaminant. Direct chemical analysis for sialic acid consistently showed the absence of this sugar in the enzyme. The fact that the carbohydrate-protein linkage is alkali-stable suggests that the linkage involves an asparaginyl-N-acetylglucosamine residue. The NH₃-terminal amino acid in the polypeptide chain is leucine.

β-glucuronidases of mammalian tissues are known to occur in a number of molecular forms (1-6). The work of Plapp and Cole (2) indicated that bovine liver contains β-glucuronidases differing in carbohydrate content, a finding consistent with the prevalent view that lysosomal enzymes are glycoproteins, with the Golgi apparatus being involved in at least some of the requisite glycosylation reactions (7). However, a major advance from the previously common assumption that microsomal β-glucuronidase is the precursor of the lysosomal enzyme derives from the elegant work of Swank and Paigen (8), who have recently presented biochemical and genetic evidence suggestive that, in the mouse, the microsomal and lysosomal forms of β-glucuronidase are independently formed from a common enzymatically active precursor, and that the microsomal forms contain varying proportions of a partner polypeptide of 50,000 molecular weight noncovalently associated with the catalytic polypeptide.

The preputial gland of the female rat is the richest known source of β-glucuronidase, this enzyme comprising 7% of the protein of the gland. Our laboratory has therefore undertaken chemical and physical studies with this enzyme, studies which would otherwise be rather difficult with enzyme available from other tissues in more limited quantity and more tedious to isolate. Keller and Touster (9) have described the preputial enzyme as a typical globular protein consisting of four identical subunits, at least in regard to the polypeptide structure. Four binding sites per molecule were also found for the potent transition state analog, saccharo-1,4-lactone (10).

Recently we have been able to resolve into at least five active components the preputial gland enzyme previously considered to be homogeneous by several criteria. Sufficient quantities of three forms were available for chemical analyses. These studies are described in the present paper.

EXPERIMENTAL PROCEDURE

Materials

Female Wistar rats (300 to 400 g) were from the exbreeder stock of Harlan Industries, Inc. Chemicals were obtained through the following sources: phenolphthalein mono-β-glucuronide, napthol-AS-BI-β-glucuronic acid, fast garnet GBC, Tris, dithiobis(nitrobenzoic acid), fluorodinitrobenzene, dansyl chloride, standard dinitrophenyl and

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1 The abbreviation used is: dansyl or Dns, 5-dimethylaminonaphthalene-1-sulfonetyl.
Dns amino acids, N-acetylenuramic acid, and mannose from Sigma; Whatman DEAE-cellulose (DE52), H. Reeve Angel and Co., Inc.: hydroxylapatite (Bio-Gel HTP), Bio-Rad Laboratories; Sephadex G-150 and Sephadex G-200 (fine), Pharmacia; bovine serum albumin, sucrose, sodium dodecyl sulfate (Catalog No. 1834), and ammonium sulfate (enzyme grade), Schwarz/Mann; p-toluenesulfonic acid, Matheson, Coleman and Bell; N-bromosuccinimide (reagent grade), Fisher, electrophoresis reagents, Canil Industries Co., etiroleurymin and ninyhydrin, Pierce; fluorodinitro[\textsuperscript{14}C]benzene, Nuclear Chicago; arabinose, glucose, galactose, and fucose, Pfannstiel Laboratories, Inc. \(\alpha\)-Acid glycoprotein was kindly supplied by Dr. Kari Schmid (Boston University), and thyroglobulin by Dr. L. W. Cunningham and Mr. John Ford (Vanderbilt University). All other chemicals were obtained commercially and they were of the highest purity available.

**Methods**

**Enzyme Assays—\(\beta\)-Glucuronidase (EC 3.2.1.31) was assayed by the standard procedure of Talalay et al. (11) as modified by Stahl and Touster (5), with the inclusion of 0.1% bovine serum albumin in the reaction mixture. One unit of \(\beta\)-glucuronidase is the amount of enzyme that catalyzes the release of 1 \(\mu\)mol of phenolphthalein/hour under standard conditions. Protein was determined by the method of Lowry et al. (17) with bovine serum albumin as standard.

**Analytical Gel Electrophoresis—**Polyacrylamide gel electrophoresis was performed under both alkaline (13) and acidic (14) conditions in a Canalco 6-place or a Buchler 12-place apparatus, at 3 mA/gel. Gels were stained for protein with Coomassie brilliant blue R250 (15). Glycoproteins were detected by the method of Zacharius et al. (16) and \(\beta\)-glucuronidase activity was detected with naphthol-AS-BI-glucuronic acid as substrate.

Split gel electrophoresis was performed by placing a plastic partition on top of the separating gel and polymerizing the stacking gel in the two resulting chambers.

**Molecular Weight Comparison—**Molecular weight comparison of the three major forms of \(\beta\)-glucuronidase was made according to Hedrick and Smith (17). Gels (4 to 7.5% polyacrylamide) were made by diluting a stock solution of 30% acrylamide (28%, w/v), methylenebisacrylamide (0.0538%, w/v). After electrophoresis (13) and staining as described above, enzyme mobility was measured relative to bromphenol blue.

**Chromatography and Gel Filtration—**Whatman microgranular DE52 cellulose and Bio-Rad hydroxylapatite were precast as recommended by the manufacturers.

**Carbohydrate Analysis—**Total carbohydrate in unhydrolyzed enzyme samples was estimated by the phenol sulfuric acid method (18), scaled down 4-fold, with mannose as standard. Sialic acid was assayed by the procedure of Warren (19) after hydrolyzing the purified enzyme for 1 hour at 80° in 0.05 M H\textsubscript{2}SO\textsubscript{4}. Standard \(N\)-acetylenuramic acid was subjected to the same acid treatment. As controls, \(\alpha\)-acid glycoprotein and thyroglobulin were assayed along with \(\beta\)-glucuronidase and consistently yielded the theoretical amount of sialic acid. Sialic acid was assayed for 1 hour at 80° in 0.05 M H\textsubscript{2}SO\textsubscript{4}, and the dansylated amino acids were extracted with polyamide sheets using the following solvent systems: (a) water-90% formic acid (260:3, v/v), (b) benzene-acetic acid (91:1, v/v), and (c) ethyl acetate-methanol-acetic acid (9:1:1, v/v).

**Amino Acid Analysis—**Amino acid analyses were performed on a Beckman model 120C amino acid analyzer. Samples (0.5 mg) were dialyzed against distilled water (8 hours), lyophilized, and hydrolyzed under vacuum at 110° ± 1° for 20 hours with 6 N HCl (2 ml) as described by Moore and Stein (23). Norleucine was used as an internal standard in all runs. Sulfur-containing amino acids were determined after performic acid oxidation according to Hirs (24). The value reported for cysteic acid was corrected assuming a recovery of 90%. Cysteine and cystine were also determined as aminomethyl-cysteine after reduction in 8 M urea and reaction with ethyleneimine according to Cole (25).

The number of free sulfhydryl groups in the enzyme was determined in 8 M urea or 0.1% sodium dodecyl sulfate in 0.1 M Tris-HCl, pH 8.0, by reaction with dithiothreitol (reprinted acid) according to Schoffen and Fontana (26).

**Protein-Carbohydrate Linkage—**Purified \(\beta\)-glucuronidase (2 mg of protein from DE52 step, Procedure A) was incubated in 0.1 N NaOH for 24 hours at 4°. Following incubation, the enzyme was passed through a Sephadex G-200 column (1.3 x 40 cm). The effluent was analyzed for protein by absorbance at 280 nm and for carbohydrate by the phenol sulfuric acid method (18).

**RESULTS**

**Preparation of \(\beta\)-Glucuronidase:** Separation of Forms A, B, and C—The \(\beta\)-glucuronidase used in the chemical studies described in this paper was prepared by one of two procedures. Procedure A is that of Keller and Touster (9), a slight modification of that of Ohtsuka and Wakabayashi (34). Procedure B, described below, yields enzyme apparently identical with that from Procedure A. It is given in detail because it was used to prepare the enzyme from which the several multiple forms were obtained by hydroxylapatite chromatography. It was developed because at one stage of our study it was important to compare the relative amounts and character of the organelle-bound enzyme in comparison to the more readily

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extractable enzyme. Procedure A involves homogenization of the preputial gland, whereas Procedure B involves extracting the enzyme from glands simply cut into very thin slices with sharp scissors. The latter operation yields an extract containing 90 to 93% of the β-glucuronidase of the gland. That the small amount of unextracted enzyme was present in lysosome-like particles was indicated by the following experiment. The slices were homogenized in 0.25 M sucrose solution, and the particulate fraction was prepared according to Carubelli and Tulsiani (35). This fraction, containing 5 to 8% of the β-glucuronidase of the gland, retained this activity on repeated washing with 0.4 M NaCl in 0.25 M sucrose. However, dialysis of the washed particles for 4 hours against ice-cold distilled water or treatment of the particles with Triton X-100 (0.1%, v/v) led to doubling of the activity and to the β-glucuronidase activity becoming nonseparable on centrifugation at 105,000 x g for 1 hour. Concurrent assays of marker enzymes for lysosomes and for other subcellular fractions confirmed that the particulate fraction containing the β-glucuronidase was highly enriched in lysosomes (data not given herein).

Purification of β-glucuronidase by Procedure B was carried out as follows. Twenty female exbreeder rats weighing 300 to 400 g were killed by decapitation. The preputial glands were quickly removed and chilled over ice. All subsequent operations were carried out at 0 to 5°. The glands, normally weighing a total of 2.5 to 3.5 g, were mixed with 6 volumes of 0.25 M sucrose solution, cut into small pieces (0.5 to 1 mm) with sharp scissors, mixed well with a glass rod, and centrifuged at 105,000 x g for 60 min. The upper lipid layer was removed with a spatula, and the supernatant solution was carefully decanted. The residue was mixed thoroughly with 6 volumes of fresh sucrose solution and centrifuged as above. The process was repeated for a third time. Ammonium sulfate was dissolved in the pooled supernatant solutions to a final concentration of 70% saturation. The suspension was kept in an ice bath for 30 min and then centrifuged at 105,000 x g for 60 min. The supernatant solution was decanted and discarded. The pellet was dissolved in 8 ml of Tris-HCl buffer (10 mM, pH 8.0) and then was dialyzed against 1 liter of the same buffer for 4 hours. The dialyzed solution was centrifuged at 105,000 x g for 60 min. The upper lipid layer was removed with a spatula, and the clear supernatant was applied to a column (2.5 x 100 cm) of Sephadex G-200. The column was eluted with 0.2 M phosphate buffer, pH 8.0. The column was then eluted with 0.1 M phosphate buffer of the same pH. The first 250 ml of eluate contained β-glucuronidase form A (Fig. 1). The column was washed extensively with the 0.1 M phosphate buffer (several hundred milliliters) until no significant amount of enzyme was found in the eluate (less than 0.01 unit/ml). Elution was then begun with 0.15 M phosphate buffer, pH 8.0. The first 250 ml of eluate contained most of β-glucuronidase form B. The column was washed with the same buffer (several hundred milliliters) until no significant amount of enzyme was detectable in the filtrate (less than 0.01 unit/ml). By similar procedures, elution with 0.2 M phosphate buffer yielded β-glucuronidase form C, and 0.25 M and 0.3 M phosphate buffers yielded two additional forms of the enzyme, but their yields were too small for further chemical investigation. An attempt to separate additional forms by using a 0.025 M phosphate step in place of a 0.05 M step after form A had been eluted was inconclusive in that 0.125 M buffer only slowly released enzymatic activity. No peak was observed, and the gradual release continued even after 1 liter of buffer had been applied.

The extensive washing of the hydroxylapatite column with each elution buffer until no further activity was removed was designed to obtain optimal separation of the enzyme forms and to preclude the possibility of chromatographic artifacts. Supporting evidence that the forms were indeed distinct was obtained by rechromatography of form C as follows: the 0.2 M phosphate eluate was diluted to 0.1 M with an equal volume of distilled water and was applied to a hydroxylapatite column (1.5 x 8 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0. Only about 5% of the enzyme activity was eluted with 50 ml of 0.15 M phosphate buffer, the remainder being eluted when the column was washed with 500 ml of 0.2 M buffer, the same concentration which eluted form C from the first column. This second hydroxylapatite column could also be used to concentrate form C by eluting the column with 30 ml of 0.3 M phosphate buffer, pH 8.0. Following hydroxylapatite chroma-

Fig. 1. Separation of molecular forms of preputial gland β-glucuronidase on hydroxylapatite columns. Enzyme (8.2 mg) purified (Procedure B) through the DE52 step was applied to a column of hydroxylapatite (1.5 x 8 cm) equilibrated with phosphate buffer (0.01 M, pH 8.0). Fraction size was 10 ml. As indicated in the text under “Results,” the breaks in the elution curves represent washing the column with several hundred milliliters of the appropriate buffer to elute completely a given enzyme form before the next higher concentration of buffer was used to elute another form of the enzyme. All buffers were pH 8.0.
tography, forms A, B, and C were concentrated to 5-ml volumes over an XM-50 membrane in an Amicon ultrafiltration apparatus.

The purification procedure summarized in Table I represents a typical experiment. Procedure B, which is very similar to Procedure A through the Whatman DE52 step, produced enzyme of the same specific activity (2200 to 2300 units/mg of protein) as Procedure A through that step. The forms separated on hydroxylapatite had slightly higher specific activity (Table I), a result suggesting that a very minor contaminant had been removed. Enzyme produced by Procedure A also yielded the several multiple forms on hydroxylapatite chromatography.

Evidence for Homogeneity of Forms A, B, and C—The purity of the three main forms of β-glucuronidase was examined by analytical electrophoresis in 7% acrylamide gels under two conditions of buffer and pH. Each form gave a single band in alkaline (13) and acidic (14) electrophoresis systems, when stained for protein, carbohydrate, or enzyme activity. Forms A and B moved identically in the former system, whereas form C moved faster than these two forms, as clearly established by the split gel electrophoresis (Fig. 2). A detailed comparison of the size versus charge relationship between different forms of β-glucuronidase was achieved by measuring their mobilities on gels of varying acrylamide concentrations as described by Hedrick and Smith (17). A plot of log Rm versus gel concentration resulted in parallel lines for forms A and C and for B and C, indicating that the forms in each pair differ mainly in their net charge and not their size. This is in agreement with the results of Sephadex G-200 gel filtration, in which the three forms were found to be eluted in the same volume of buffer.

Comparison of Properties of Forms B and C of β-Glucuronidase—Because form C was found to contain about twice the carbohydrate (see below) as forms A or B, some comparison of their properties seemed desirable. Forms B and C were compared in the following experiments: (a) kinetic studies, the two forms gave the same K_m values (9 × 10^{-5} M) for phenolphthalein glucuronide; (b) stability, the two forms were very stable in Tris-HCl buffer, pH 8.0, at 0 to 4°C, little loss of activity being observed over a 30-day period. In sodium acetate buffer, pH 5.0, both forms lost most of their activity in several days in the cold room. When the standard assay was run for 10 min at varying temperatures, both forms exhibited an optimum at 70°C, the activity being 2.7 times that at 37°C. However, when the enzymes were heated at 70°C prior to assay, complete inactivation occurred in 10 min.

Amino Acid Composition—Enzyme resolved into three components by Procedure B gave the analyses shown in Table II. No significant differences among the forms are evident. The three different methods used to determine tryptophan content in enzyme purified by Procedure A gave the following results (expressed as residues per subunit): spectrophotometry, 14; N-bromosuccinimide titration, 15; hydrolysis in p-toluensulfonic acid, 12. Although these values are in fair agreement, they do not permit unequivocal designation of the number of tryptophyl residues per subunit. The two different methods used to determine cysteine and cystine in the Procedure A preparation gave the following values (in residues per subunit): performic acid oxidation, 5.4; tetrationation, 5.6. Titration of β-glucuronidase with dithiobis(nitrobenzoic acid) in the presence of 8 M urea or 0.1% sodium dodecyl sulfate gave no —SH-positive reaction results, suggesting that there are 12 (+4) disulfide bonds per tetramer. These disulfide bonds are apparently intrachain, because electrophoresis in sodium dodecyl sulfate without the addition of mercaptoethanol to the preincubation mixture or gels yielded a band which migrated to the same position as a sample treated with mercaptoethanol.

It was also found that after treatment with dithiobis(nitrobenzoic acid) and subsequent dilution and assay, full activity was recovered. This result is in contrast to the work of Fernley (36), who concluded that, based on heavy metal inactivation, preputial gland β-glucuronidase has a sulfhydryl at its active site, a conclusion also inconsistent with the work of Wang and Touster (37, 38) on the active site of rat liver lysosomal β-glucuronidase. Dixon and Webb (39) have mentioned that heavy metals might react not only with sulfhydryl

![Fig. 2. Analytical polyacrylamide gel electrophoresis (7% gel) of preputial gland β-glucuronidase (Procedure B) by the method of Davis (13). The gel at the far right is a split gel. Electrophoresis was carried out as described under "Methods." The bands shown were obtained by protein stain (3), with 5 to 10 μg of each enzyme form applied to the gels, or by β-glucuronidase activity stain (3), for which only 75 milliunits of enzyme were subjected to electrophoresis.](http://www.jbc.org/)

| Table I

| Purification of β-glucuronidase from female rat preputial glands (procedure B) |
|-----------------------------|-----------------|----------------|-----------------|-----------------|-----------------|
| Fraction                   | Protein (mg)    | Yield (%)      | Enzyme activity (units) | Yield (%) | Specific activity (units/mg) | Purification ratio |
|-----------------------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|
| Gland secretion            | 176.0           | 100.0          | 34,460          | 100.0           | 196             | 1.0             |
| (NH_4)_2SO_4 (0 to 70%) saturation and dialysis | 90.5           | 51.4           | 31,960          | 92.7            | 353             | 1.8             |
| Sephadex G-200 gel filtration | 15.5             | 8.8            | 26,840          | 77.9            | 1,730           | 8.8             |
| DE52 chromatography        | 8.2             | 4.7            | 18,430          | 53.5            | 2,250           | 11.5            |
| Hydroxylapatite chromatography | 0.87           | 0.5            | 2,120           | 6.2             | 2,440           | 12.4            |
| β-Glucuronidase A          | 2.60            | 1.5            | 6,200           | 18.0            | 2,390           | 12.2            |
| β-Glucuronidase B          | 1.00            | 0.7            | 2,540           | 7.4             | 2,540           | 13.0            |
Tyrosine
Threonine
Arginine
Aspartic acid
Threonine
Serine
Glutamic acid
Proline
Glycine
Alanine
Valine
Methionine
Isoleucine
Leucine
Tyrosine
Phenylalanine

NH$_2$-terminal Amino Acid—In both the dansyl and fluorodinitrobenzene methods, the only NH$_2$-terminal amino acid found in β-glucuronidase purified (Procedure A) through the DE52 step was leucine, a result indicating that the multiple forms have the same amino terminal residue.

Carbohydrate Composition—β-Glucuronidase subjected to electrophoresis on polyacrylamide gels stained for both protein and carbohydrate as expected from previous reports (2, 5) indicating that this enzyme is a glycoprotein. Analyses for sugars were carried out on samples from the Whatman DE52 column and on the three forms obtained by chromatography on hydroxylapatite (Table III). The analyses indicate that forms A and B are very similar in carbohydrate content, whereas form C is much richer in sugars. The gas-liquid chromatography analysis for individual sugars agrees with the total sugar method on this point. The absence of sialic acid is noteworthy, because Goldstone and Koenig (40, 41) have presented indirect evidence that glucose, as well as the other hexoses, is an integral component of the enzyme. First, the large volume of 0.2 M phosphate buffer from the hydroxylapatite column after most of form C had been eluted was concentrated and subjected to hydrolysis and gas-liquid chromatography analysis as described under “Methods.” The mannose, fucose, galactose, and glucose contents (per mg of protein) were essentially the same as for form C itself. Second, form C was dissociated with 8 M urea at pH 5.0, applied to a Sephadex G-150 column equilibrated with the urea solution, and eluted with the same urea solution. Fractions containing enzyme activity were pooled, dialyzed against distilled water for 2 days, concentrated to a small volume, and subjected to gas-liquid chromatography analysis for the four neutral sugars. Again, they were present in their usual amounts. Finally, in view of the finding of Rudick and Elbein (42) that an extracellular β-glucosidase from Aspergillus fumigatus purified to apparent homogeneity contains a large amount of glucose which is completely removed by trichloroacetic acid precipitation of the enzyme, the preputial gland β-glucuronidase (DE52 product) was precipitated in 5% trichloroacetic acid in the cold and then the protein precipitate was washed, hydrolyzed, and analyzed as above. The ratios of the four sugars were similar to those usually found in the purified enzyme (DE52 product). Taken together, these three experiments leave little doubt that the preputial enzyme is not contaminated by carbohydrate-rich substances and, more specifically, that glucose is present essentially in the quantities shown in Table III. The identity of the four neutral sugars routinely determined by gas-liquid chromatography was confirmed by paper chromatography, and the presence of glucosamine, rather than galactosamine, was established by analysis on an amino acid analyzer (see “Methods”).

Attachment of Carbohydrate to Polypeptide—Following treatment of the enzyme with 0.1 N NaOH (see “Methods”) all of the phenol sulfuric acid-positive material was found to co-elute from Sephadex G-200 with the protein. The alkali stability of the linkage indicates that the carbohydrate is linked to the protein via an asparaginyl N-acetyl-glucosamine linkage, as is common in glycoproteins containing mannose and glucosamine as the principal sugars.

**DISCUSSION**

Although previous studies have yielded evidence for the occurrence of several molecular forms of β-glucuronidase,
extensive carbohydrate analyses on such forms have not been reported. The most relevant previous paper in this regard is that of Plapp and Cole (2), who analyzed several forms of beef liver \( \beta \)-glucuronidase and found similar amino acid composition but different total carbohydrate content. These and many other investigators have employed an autolytic step which of course might have altered the composition of glycoproteins. Knowledge of the chemistry of the multiple forms of glycoprotein enzymes obtained from a given tissue should contribute to our understanding of the biosynthetic steps and the transport processes for these enzymes. Moreover, the pioneering studies of Ashwell, Morell and their co-workers (43), as well as contributions by Rogers and Kornfeld (44) and from Neufeld’s laboratory (45), have established that the nature of the terminal sugars in glycoproteins at least in part determines the extent to which these macromolecules are taken up by cells.

Very relevant to the present report is the demonstration by Brot et al. (46) that \( \beta \) glucuronidase preparations from different human tissues exhibit dissimilar rates of uptake by human fibroblasts. These uptake studies were initiated because of the interest in the therapy of patients with atypical mucopolysaccharidosis, a condition apparently resulting from a deficiency of lysosomal \( \beta \)-glucuronidase (47, 48).

The amino acid analyses of the three forms reported here are very similar to each other and are in substantial agreement with the analysis of the rat liver lysosomal form reported by Stahl and Touster (5). This result is not only consistent with the evidence that the enzymes from the two organs have the same, or very similar, native molecular weight (9, 9) but also with the similar reactivity of the two enzymes toward antibody prepared against the preputial gland enzyme (49). On the other hand, the results reported herein differ significantly for several amino acids from those published by Ohtsuka and Wakabayashi (34) for the preputial gland enzyme. The explanation for these discrepancies is unknown, although a difference in properties of the enzyme prepared in the two different laboratories has been discussed (9). The present results also show a close similarity in amino acid composition of the preputial gland enzyme to the bovine liver enzyme (50) but, in contrast, not to the human liver enzyme (51).

The carbohydrate composition of the preputial gland \( \beta \)-glucuronidase is of special interest. The presence of sialic acid in lysosomal enzymes has been inferred from neuraminidase-induced changes in electrophoretic mobility (40, 41) and in rabbit liver \( \beta \)-glucuronidase from similarly induced effects on isoelectric points (6). Direct chemical analysis of the preputial enzyme demonstrated the absence of this sugar not only in the preputial gland enzyme, which is a secretory product, but also in pure mouse liver lysosomal \( \beta \)-glucuronidase. The need for special caution in the use of neuraminidase even when it is purified by affinity chromatography is highlighted by the recent report of Rood and Wilkinson (52).

The finding of glucosamine and mannose as the major sugars is consistent with the widespread occurrence of these two sugars in mammalian glycoproteins. In regard to the occurrence of fucose in this secreted form of \( \beta \)-glucuronidase, it is relevant to mention that Bennett et al. (53) have presented autoradiographic evidence, obtained in the rat, for the addition of fucose to glycoproteins in the Golgi apparatus before these proteins are incorporated into secretory vacuoles, lysosomes, and plasma membranes. (However, note comment below on the absence of fucose in mouse liver lysosomal \( \beta \)-glucuronidase.)

Two other sugars, galactose and glucose, were found to be constituents of \( \beta \)-glucuronidase. In early experiments reported previously (54, 55), galactose was found only in variable, trace amounts, and glucose was not observed at all even though the same methods for the hydrolysis and gas-liquid chromatography analysis have been used continuously in our laboratory. Although the reason for these variations cannot be established at this time, we favor the view that an altered physiological state of the rats is responsible. The preputial gland of the female rat, a vestigial organ of undefined, perhaps, nonessential role, has been shown by Szego et al. (56) to respond to endocrinological influences. Hormonal effects on specific \( \beta \)-glucuronidase isozymes in this gland have also been reported (57).

Only recently has convincing evidence been amassed for the presence of covalently bound glucose in mammalian glyco proteins (58-61), other than those of the collagen-basement membrane type. The several special experiments described under “Results” would appear to demonstrate conclusively that glucose, as well as galactose, is present in \( \beta \)-glucuronidase produced in the preputial gland of the animals presently available. Examination of the molar ratios of sugars per unit of enzyme indicates the occurrence of heterogeneity among the subunits or among the carbohydrate clusters within the subunits. The ratios for mannose to glucosamine to fucose to galactose to glucose were as follows: form A and B, 10:7:0:8:0:6:0:6; form C, 13:7:1:4:6:4:5. Although not tested for directly, the glucosamine may be presumed to be N-acetylated (62).

Although forms A and B of the preputial enzyme are separable on hydroxylapatite columns, the nature of the structural difference between the two remains to be established. Within experimental error, they have yielded the same amino acid and carbohydrate composition. Refinement of our analytical procedures might disclose a difference. Alternatively, there may be an unknown constituent present, or one or more residues of the acidic amino acids may be present as the amide. Form C obviously differs from A and B mainly in carbohydrate, although very minor differences in the polypeptide portion are not positively precluded. Because at least four of the five sugar constituents are elevated in form C as compared to A and B, it may be concluded that the differences are not due simply to the presence of a small number of terminal sugar residues. It should be noted that the largest differences are in fucose, galactose, and glucose, rather than in glucosamine or mannose. Indeed, glucosamine shows little variability among the forms. It is likely that this sugar is located at the more internal regions of the molecule, an assumption consistent with an analysis of mouse liver \( \beta \)-glucuronidase, which has the same glucosamine content as the rat preputial enzyme but very different amounts of mannose, galactose, and glucose, and no fucose (or sialic acid).²

Before the hydroxylapatite column was developed for the separation of the different forms of the enzyme, glycopeptides were prepared from preputial gland \( \beta \)-glucuronidase purified through the DE52 step, by repeated pronase digests followed by Sephadex G-25 gel filtration. The purified glycopep-

¹S. Tomino, K. Paigen, D. R. P. Tulsiani, and O. Touster, manuscript in preparation.

²R. K. Keller and O. Touster, unpublished work.
tide preparation contained 5.7 mol of mannose, 2.2 mol of glucosamine, 0.41 mol of fucose, and 1.0 mol of aspartic acid/mole of NH₂-terminal estimated by the trinitrobenzoic acid method of Fields (63). This result suggested that the carbohydrate is present in an average of two to three clusters per polypeptide subunit. Ion exchange chromatography of the glycopeptide preparation on Dowex 50-X2 yielded several peptides varying greatly in mannose content and in fucose as well, but containing approximately 2 mol of glucosamine per NH₂-terminal group. These results are consistent with the present findings that the different molecular forms of β-glucuronidase vary little in glucosamine content, but they require confirmation from the analysis of glycopeptides prepared from the separated molecular forms of the enzymes. The involvement of a chitobiose unit linking mannose to an asparagine residue appears to be a common glycoprotein structural feature (42, 64, 65). In addition, a range in mannose content in glycopeptides isolated from thyroglobulin has been reported (65).

The finding of appreciable differences in sugar content in the forms of β-glucuronidase may find application in the study of the influence of sugar composition on the rate of uptake of these forms by fibroblasts.

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