Purification and Characterization of Mouse Kidney \( \beta \)-Glucuronidase

(Received for publication, October 14, 1974 )

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\( \beta \)-Glucuronidase has been purified from mouse kidneys previously induced by gonadotrophin to a specific enzyme activity 15 times higher than the non-induced kidney. The purification procedure includes ultrasonication to solubilize the enzyme, acid and ammonium sulfate precipitations, gel filtration in Sephadex G-200, DEAE-ion exchange chromatography, and isoelectric focusing. The resulting product has a specific activity of 284,000 Fishman units/mg of protein, representing a 1,090-fold purification and is 17,000-fold higher than the level in the non-induced kidney. The purified \( \beta \)-glucuronidase is apparently homogeneous by criteria of gel filtration, sodium dodecyl sulfate gel electrophoresis, and immunodiffusion. Characterization of the purified enzyme showed that it is identical with the lysosomal isoenzymic form electrophoretically, has subunit molecular weight of 74,000 (estimated by sodium dodecyl sulfate gel electrophoresis) and oligomer molecular weight of 300,000. The purified enzyme is stable at high temperature (up to 55\(^\circ\)C) and at wide range of pH (from 4 to 11). It has a pH optimum for its activity at 4.7 and a \( K_m \) of \( 1.18 \times 10^{-4} \) M. The purification and characterization of this enzyme from mouse kidney will have significance in the understanding of the molecular nature of the isoenzymes of \( \beta \)-glucuronidase and will be useful in future studies on the mechanism of intracellular transport and distribution of this hydrolase.

Among various systems used for the study of \( \beta \)-glucuronidase (EC 3.2.1.31), the enzyme in mouse kidney has been studied the most actively primarily because in this tissue it can be induced specifically by androgenic hormones (1) as a result of increase in de novo synthesis of the enzyme protein (2, 3). Thus, Kato et al. (4, 5) from their observation of the temporal sequential increase of \( \beta \)-glucuronidase in various subcellular sites after gonadotrophin injection, suggested that the newly synthesized enzyme was transported from the membrane-bound ribosome across the membrane into the cisternae of rough endoplasmic reticulum and then into the lysosome via smooth endoplasmic reticulum. Marsh et al. (6) further demonstrated the involvement of Golgi apparatus in this transport.

This enzyme, even though generally identified as one of the lysosomal acid hydrolases, is distributed in substantial amounts in extralysosomal sites (6-8) as in mouse kidney, where about 30 to 40\% of the activity is present in the microsomal fraction (9, 10). Genetic studies by Paigen (11) have shown that a single gene codes for this enzyme in both lysosomal and microsomal fractions. Now, the inducibility of this enzyme in the kidney of different strains of inbred mice has qualified it as a model system for the study of control of gene action in eukaryotes (3, 12).

Multiple enzyme forms of \( \beta \)-glucuronidase have been found in bovine liver (13), rat liver (14, 15), and more recently in mouse kidney (16, 17) by chromatography and electrophoresis. Swank and Paigen (16) have shown that the microsomal \( \beta \)-glucuronidase of mouse kidney exhibited five distinct electrophoretic components different from the lysosomal one. It was suggested (16) that the existence of one to four accessory polypeptide chains attached to the primary structure of the enzyme protein is the cause of the isoenzymic forms of microsomal \( \beta \)-glucuronidase.

A recent report by Lin (18) has now shown that the single lysosomal enzyme can be separated into four components by isoelectric focusing in polyacrylamide gels. The possible cause of the multiple forms of the lysosomal enzymes of bovine and rat livers (13, 14) is attributed to the carbohydrate content of the enzyme. Goldstone and Koenig (19) suggested that acid hydrolases, including \( \beta \)-glucuronidase, are modified by the addition of acid carbohydrate moieties to the enzyme protein during the transport from endoplasmic reticulum to lysosomes. Swank and Paigen (16), however, suggested that the formation of the lysosomal enzyme is independent of the microsomal forms and both are formed independently from the structural gene product. The molecular nature of the various isoenzyme forms existing in different organelles, therefore, becomes important in efforts to elucidate the processes of the formation and intracellular transport of \( \beta \)-glucuronidase and other lysosomal hydrolases.

Nevertheless, \( \beta \)-glucuronidase of the mouse kidney has never been purified to homogeneity. The highest specific activity reported was that by Pettengill and Fishman (20) who obtained
a preparation with the methods available 12 years ago of 81,000
Fishman units/mg of protein. The maximum specific activity
obtained in the isotope incorporation study of Kato et al. (4)
was 76,000 units/mg of protein. Higher specific activities have
been recorded for presumably homogeneous /3-glucuronidase
preparation of bovine liver, rat liver lysosomes, rat preputial
gland, and rabbit liver (21, 25).

The purpose of the present study is, therefore, to obtain
mouse kidney /3-glucuronidase in a state of homogeneity so that
the physicochemical and enzymatic properties of this enzyme
can be characterized. These data are essential for our further
studies in verifying the routes of intracellular transport of this
enzyme and the understanding of the molecular nature and
 genetic relationship of various isoenzyme forms of /3-glucuronidase
which are of fundamental concern to other laboratories.

The preparation of this enzyme in a high degree of purity
from mouse kidney is probably hindered by both the small size
of the organ and the relatively low activity of the enzyme. By
taking advantage of the inducibility of the enzyme by andro-
genic hormones, we were able to enrich the mouse kidneys to an
extent which made possible the purification of the enzyme by
column chromatography and isoelectric focusing techniques to
a stage of apparent homogeneity. In this paper, we report the
procedures and the results of the purification, the tests of
purity, and the characterization of the purified enzyme.

EXPERIMENTAL PROCEDURES

Animals—Male A/Jax mice, about 2% months old, obtained from
Jackson Laboratory (Bar Harbor, Me.) were used for the study.
Animals were injected intraperitoneally daily for 8 days with 10 i.u. of
chorionic gonadotrophin (Follstein, E. R. Squibb & Sons, Inc., New
York) in 0.25 ml of saline (0.9% NaCl solution) per animal to induce
the enzyme activity in the kidney.

Materials—Chemicals of the highest available purity were obtained
through the following sources: phenolphthalein P-glucuronide, synthe-
sized and recrystallized in our laboratory by using the bosynthesis
method described by Fishman (26); naphthol AS-BI glucuronide,
catalase, leucine aminopeptidase were obtained from Sigma Chemical
Co.; bovine serum albumin and ferritin from Nutritional Biochemical
Co.; rabbit muscle phosphorylase A and thyroglobulin from Schwarz/
Mann; aldolase from Pharmacia Chemical Co.; and Ampholine for
isoelectric focusing from LKB Instruments, Inc. All other chemicals
were reagent grade purchased from various commercial sources.

Assay Procedures—/3-Glucuronidase activity was determined by the
method described by Fishman (26) using phenolphthalein /3-glucosidu-
ronic acid (1 mm) as substrate in 0.1 M acetate at pH 5.2. Unit of
enzyme activity is defined as micromol of phenolphthalein produced
per hour of incubation (Fishman unit).

Protein concentration was determined by the method of Lowry et al.
(27).

Production of Antibody—Anti-/>-glucuronidase was produced by
injecting the purified enzyme intradermally into a rabbit with 0.125
mg of the enzyme protein in 0.5 ml mixed with an equal volume of
complete adjuvant (Freund, Difco Laboratories). Two booster injec-
tions with half of the quantity of antigen were given at weekly intervals
after the original injection. Blood samples were collected from the
rabbit at weekly intervals 2 weeks after the initial injection. Immuno-
diffusion tests showed that the antiserum obtained 4 weeks after the
initial antigen injection and diluted 3-fold, reacted optimally with the
purified enzyme, at the activity of 71,000 units/ml.

Immunodiffusion—The precipitin test for the antibody-antigen
reaction was carried out in agar gel following the double diffusion
procedure of Ouchterlony (28). The enzyme samples and the antiserum
were allowed to diffuse at 4° in the agar gel (1.5% in 0.05 M Veronal
buffer, pH 8.6) for several days until precipitin lines were visible. The
gel was then washed in several changes of saline to remove unreacted
components. The enzyme-antibody complex, which is enzymatically
active, is visualized by the same staining method used for the
electrophoretic gels.

Electrophoresis of /3-Glucuronidase—Electrophoretic studies of the
enzyme were carried out according to the method described by Clarke
(29) and by Swank and Paigen (18). Polycrylamide gels (75%, 4 × 90
mm) were prepared in Tris-glycine buffer, pH 8.5. Samples were
extracted with 5% Triton X-100 in 0.25 M succrose and 0.02 M imidazole
buffer (pH 7.4) and centrifuged at 105,000 × g for 1 hour to obtain the
clear supernatant. Ten micromolar of these extracts were then applied
to each gel for electrophoresis, which was carried out in Tris-glycine
buffer, pH 8.1, and at a constant voltage of 200 volts for 90 min. It is
essential that the gel be cooled by cold water circulation to reduce the
heat generated during electrophoresis, since change of isoenzyme forms
of /3-glucuronidase can occur from heating (16, 18).

After electrophoresis, gels were preincubated at 0.2 M acetate buffer
(pH 5.2) for 30 min before being stained for /3-glucuronidase. The
procedure for staining of the enzyme in the gel was adapted from the
histrochemical method described by Hayashi et al. (30). Naphthol
AS-BI glucuronide (0.25 mm) was used as substrate. The staining
reaction was carried out at pH 5.2 in 0.1 M acetate buffer. The reaction
product was coupled simultaneously with 0.3 mm hexazonium pararos-
amnin to produce pink color bands where enzyme activity was located.

Molecular Weight Determinations—Subunit molecular weight was
estimated by electrophoresis in sodium dodecyl sulfate polyacrylamide
gels by the method of Fairbanks et al. (31). Aldolase (subunit MW
39,500), catalase (subunit MW 60,000), bovine serum albumin (MW
67,000), and phosphorylase A (subunit MW 94,000) were used as
molecular weight standards. Proteins were incubated with the sodium
dodecyl sulfate-mercaptoethanol-Cleland reagent solution (31) for 45
min at 45°. Electrophoresis was carried out in 7% acrylamide gel at a
constant current of 7 ma per gel (0.6 × 8.5 cm) for 2 hours at room
temperature with pyronin Y as tracking dye. Gels were stained for
protein with Coomassie blue and the relative migration rate of each
protein was calculated against the migration of the tracking dye.

Molecular weight of the /3-glucuronidase oligomer was determined
by electrophoresis in 4 to 30% polyacrylamide gradient gels (32),
supplied by Pharmacia Fine Chemicals, Inc. The electrophoresis was
 carried out in Tris-borate buffer (90 to 80 mM) with 3 mM EDTA at pH
8.35 for 40 hours with a voltage maintained at 125 volts. Thyroglobulin
(MW 670,000), ferritin (MW 450,000), phosphorylase A (MW 370,000),
leucine aminopeptidase (MW 300,000), and catalase (MW 240,000) were
used as molecular weight standards.

RESULTS

Induction of Mouse Kidney /3-Glucuronidase by
Gonadotrophin

The result of induction of /3-glucuronidase by daily injection of
gonadotrophin (10 i.u./mouse/day) is shown in Fig. 1. The
specific enzyme activity reached its maximum at about 7 to 9
days of the experiment and represented a 13-fold increase of
activity from the non-induced level (210 versus 16.7 units/mg
of protein). The maximum activity obtained in this experi-

![Fig. 1. Response of mouse kidney /3-glucuronidase to daily adminis-
tration of gonadotrophin (10 i.u./mouse/day). Enzyme activity is
expressed as micromolar of phenolphthalein released per hour (Fish-
man unit). Maximum specific activity occurred at between 7 to 9 days.](http://www.jbc.org/content/250/14/4738/F1)

Downloaded from http://www.jbc.org/ on October 13, 2017
(42,000 units/g of kidney) is approximately equal to the highest reported in the literature for this enzyme in hormone-induced kidney (33, 34). The rapid fall in enzyme level after 9 days is probably due to exhaustion of the testis mechanism for synthesizing testosterone.

**Purification of β-Glucuronidase**

**Step I: Homogenization**—Eighty-nine mice, after receiving daily gonadotrophin injections for 8 days, were killed by decapitation. The kidneys, 31.4 g, were homogenized in 10 mM Tris-HCl buffer, pH 7.0, with 10 mM MgCl₂ in a Waring blender for 1 min to form a 20% (w/v) homogenate. The specific activity of this crude homogenate was 259 units/mg of protein, which represented a 15.5-fold induction of the enzyme activity by the hormone.

**Step II: Ultrasonication**—This and subsequent steps were done at 4°C. To release β-glucuronidase from lysosomes and the microsomal membranes, the homogenate was sonicated by passing it through a continuous flow chamber of an ultrasonicator (Bronwill Biosonic IV) setting at 80% of the maximum intensity. The flow rate of the sample through the chamber (5-ml capacity) was adjusted to 2.5 ml/min. After sonication, the homogenate was centrifuged at 105,000 x g for 30 min. The sediment was washed once with 10 mM acetate buffer, pH 5.0, and the supernatant combined.

**Step III: Acid Incubation**—The clear supernatant from Step II was adjusted to pH 5.0 with 0.4 M acetic acid and incubated for 3 hours at 37°C, then centrifuged at 48,000 x g for 30 min. The sediment was washed once with 10 mM acetate buffer, pH 5.0, and the supernatant combined.

**Step IV: Ammonium Sulfate Precipitation**—The supernatant from Step III was readjusted to pH 7.0 with 0.1 M NaOH and solid (NH₄)₂SO₄ was added to 55% saturation with continuous mixing. The mixture was allowed to stand overnight before being centrifuged at 48,000 x g for 20 min. The precipitate was resuspended in 10 ml of 10 mM Tris-HCl buffer, pH 7.0.

**Step V: Gel Filtration in Sephadex G-200**—The product from the previous step (10 ml) was applied to a Sephadex G-200 column (5 x 90 cm) equilibrated with 10 mM Tris-HCl buffer at pH 7.0, and eluted with the same buffer. As shown in Fig. 2, the enzyme was eluted out as a single peak before the bulk of the protein. The pooled peak fractions contained 77% of the enzyme activity applied.

**Step VI: DEAE-Sephadex Chromatography**—The pooled enzyme fractions from the Sephadex column (204 ml) were applied to a DEAE-Sephadex A-50 column (1.5 x 25 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0), washed with 100 ml of the Tris buffer, and then eluted with a linear gradient of 0.1 to 0.3 M NaCl in the same buffer. The enzyme appeared at about 0.2 M NaCl as a symmetrical peak (Fig. 2).

**Step VII: First Isoelectric Focusing**—The pooled enzyme fractions of the DEAE-Sephadex column were dialyzed against 10 mM Tris buffer (pH 7.0) and concentrated by lyophilization. This was then applied to an isoelectric focusing column (LKB Instrument Co., 110-ml capacity) with pH gradient of 5 to 7 provided by 2% Ampholine and stabilized by sucrose gradient (0 to 40%, w/v). The isoelectric focusing was carried out at a constant voltage (500 volts) for 45 hours. At the completion of the run, the column was emptied and 1-ml fractions were collected. The enzyme activity was focused at pH 5.8 which corresponded to the protein peak located at the same pH region (Fig. 3). The yield of this step was 73%. The specific activity can not be accurately estimated due to the presence of Ampholine which formed a strong blue color in the Lowry protein determination procedure. However, in this purification procedure, a considerable amount of nondenzyymatic protein had focused at pH below 4 and was removed as precipitate.

**Step VIII: Repeat Isoelectric Focusing**—The enzyme peak from the first isoelectric focusing column was refocused at a pH gradient of 5 to 7. One major protein peak was obtained which corresponded to the enzymatic activity (Fig. 3).

**Fig. 2.** Purification of β-glucuronidase by Sephadex G-200 gel filtration (upper graph) and DEAE-Sephadex chromatography (lower graph). In gel filtration, 10 ml of sample solution in 10 mM Tris-HCl buffer (pH 7.0) was applied to the Sephadex G-200 column (5 x 90 cm) and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 10 ml were collected. In DEAE-Sephadex chromatography (lower graph), the enzyme fractions from the Sephadex G-200 column were applied to a DEAE-Sephadex A-50 column (1.5 x 25 cm) at pH 7.0 maintained by 10 mM Tris buffer. After washing with 100 ml of the same buffer, a linear NaCl gradient of 0.1 to 0.3 M in the Tris buffer was applied to elute out the enzyme. Fractions of 5 ml were collected.

**Fig. 3.** Isolelectric focusing of mouse kidney β-glucuronidase. The dialyzed and concentrated enzyme fractions from DEAE-Sephadex column was applied to an isoelectric focusing column (LKB Instrument Co., 110 ml capacity) in 2% Ampholine, pH 5 to 7 (left graph). The isoelectric focusing was carried out at a constant voltage of 500 volts for 45 hours. Fractions of 1 ml were then collected and enzyme activity, pH, and absorbance at 280 nm were measured. The enzyme fractions were pooled and refocused under the same conditions (right graph). One major protein peak was observed which corresponded to the enzymatic activity focused at pH 5.8.
Step IX: Repeat Gel Filtration on Sephadex G-200—The enzyme fractions from the second isoelectric focusing step were pooled, dialyzed against 10 mM Tris buffer (pH 7.0) to remove most of the Ampholine and sucrose and concentrated by lyophilization. This was then applied to a Sephadex G-200 column (2.5 x 92 cm) and eluted with the Tris buffer. A single protein peak was recovered which corresponded with the enzymatic activity (Fig. 4). The peak fractions were pooled and concentrated by lyophilization and dissolved in 3.1 ml of Tris buffer. The entire purification scheme is summarized in Table I. The overall purification was 1,090-fold with 13.7% recovery of the enzyme. The specific activity of the final preparation was 284,000 Fishman units/mg of protein, which was about 17,000 times higher than the enzyme level of non-induced mouse kidney (16.7 units/mg of protein).

Test for Purity of Enzyme Preparation

Electrophoresis in Sodium Dodecyl Sulfate Polyacrylamide Gels—The protein patterns of the enzyme preparations in several steps of the purification were examined by electrophoresis in sodium dodecyl sulfate gels. As shown in Fig. 5, the enzyme became a recognizable protein band after the (NH₄)₂SO₄ precipitation step and was the major component after the DEAE-Sephadex chromatography. The final preparation exhibited a single band in the sodium dodecyl sulfate gel. The purity of the final enzyme preparation was also tested by increasing the amount of protein applied to the sodium dodecyl sulfate gels. As shown in Fig. 5, a single protein band was observed at up to 8 µg of enzyme protein applied.

Immunodiffusion—The purity of the purified enzyme was also tested by double immunodiffusion in Ouchterlony plates. The result is shown in Fig. 6 in which the antibody to the final preparation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Volume (ml)</th>
<th>Enzyme (10⁶ units)</th>
<th>Protein (mg)</th>
<th>Specific Activity (unit/mg protein)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude homogenate</td>
<td>160</td>
<td>1,600</td>
<td>6,180</td>
<td>259</td>
<td>100</td>
<td>1</td>
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<tr>
<td>2</td>
<td>Ultrasonicated supernatant</td>
<td>218</td>
<td>1,434</td>
<td>2,746</td>
<td>537</td>
<td>92</td>
<td>2.1</td>
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<tr>
<td>3</td>
<td>Acid incubation &amp; centrifugation</td>
<td>244</td>
<td>1,371</td>
<td>1,318</td>
<td>1,040</td>
<td>86</td>
<td>4.0</td>
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<tr>
<td>4</td>
<td>(NH₄)₂SO₄ precipitation</td>
<td>101</td>
<td>901</td>
<td>277</td>
<td>3,253</td>
<td>56</td>
<td>12.6</td>
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<tr>
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<td>Gel filtration on Sephadex G-200</td>
<td>204</td>
<td>680</td>
<td>69.4</td>
<td>9,891</td>
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<td>6</td>
<td>Chromatography on DEAE-Sephadex A-50</td>
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<td>31.7</td>
<td>20,577</td>
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<td>First isoelectric focusing</td>
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<td>8</td>
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<td>Gel filtration on Sephadex G-200 lyophilization</td>
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<td>0.77</td>
<td>294,790</td>
<td>13.7</td>
<td>1090</td>
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</table>

*Not available due to the presence of ampholine which interferes with the Lowry protein determination procedure.
ENZYME ACTIVITY

FIG. 6. Immunodiffusion of the β-glucuronidase. The antibody was prepared by injecting the purified enzyme into a rabbit. The center well contained the antiserum, diluted 1:1000. The surrounding wells: A, contained the purified enzyme (diluted to 1,400 units/ml); B, enzyme preparation after the isoelectric focusing; C, after DEAE-Sephadex chromatography; D, after Sephadex G-200 column; E, after ammonium sulfate precipitation; and F, the sonicated supernatant. One single precipitin line was formed which was completely confluent for all the enzyme preparations (left graph). Enzyme stain shows that the activity corresponds to the precipitin line (right graph).

FIG. 7. Molecular weight estimations of subunit (upper graph) and oligomeric β-glucuronidase (lower graph). Sodium dodecyl sulfate gel electrophoresis of Fairbanks et al. (31) was used for molecular weight estimation of the enzyme subunit. Aldolase (subunit MW 39,500), catalase (subunit MW 69,000), bovine serum albumin (BSA) (MW 67,000), and phosphorylase A (subunit MW 94,000) were used as molecular weight standards. The result which is the average of four separate runs, yields a value of 74,000 (ranging from 73,000 to 75,000) for the molecular weight of β-glucuronidase subunits.

The error contributed to the sodium dodecyl sulfate gel molecular weight estimation by 3 to 6% of carbohydrate content in the glycoprotein is usually within the experimental error, although higher amounts of carbohydrate do influence the reliability of the sodium dodecyl sulfate data. Since Plapp and Cole (21) reported that bovine liver β-glucuronidase contains 3 to 6% carbohydrate, we are inclined to accept the subunit molecular weight data estimated in this paper for the mouse kidney enzyme.

Molecular weight determination of the β-glucuronidase oligomer was performed by electrophoresis in linear gradient polyacrylamide gels, from 4 to 30% concentration. The result (Fig. 7) showed that the molecular weight of the β-glucuronidase purified from mouse kidney is 300,000.

These results are different from those obtained by Swank and Paigen (16) who estimated by polyacrylamide gel electrophoresis that the basic tetrameric enzyme structure has a molecular weight of 260,000 with subunit molecular weight of 65,000. However, the values obtained in the present study were in closer agreement with those obtained by Stahl and Touster (22) from rat liver lysosomes and by Plapp and Cole (13) from bovine liver; all reporting the molecular weight of β-glucuronidase at around 280,000.

Properties of Purified Mouse Kidney β-Glucuronidase

Electrophoretic Patterns—The electrophoretic patterns of β-glucuronidase during the purification (Fig. 8) showed that the purified enzyme is identical with the lysosomal component L, as obtained by Swank and Paigen (16) and our previous observation (18). The lysosomal (L) and three of the microsomal components (M1, M2, and M3) are visible in the

1B. S. Leach and W. W. Fish, 168th American Chemical Society Meeting, September 9 to 13, 1974; and B. S. Leach, personal communication.
zymogram of the crude homogenate. After the ultrasonication step, only the lysosomal component is visible. Since it is known that the microsomal components readily convert to the lysosomal component under various conditions (16, 18) and that 92% of the enzyme activity is recovered in the ultrasonication step, it is most likely that this conversion might have taken place during ultrasonication.

Effect of Substrate Concentration—The effect of substrate concentration on enzyme activity assayed at pH 5.2 is seen in Fig. 9. The reaction appears to obey normal Michaelis-Menten kinetics and has a $K_m$ value of $1.18 \times 10^{-4} \text{M}$. At the lowest substrate concentration tested (0.05 mM), about 2% of the substrate was hydrolyzed in 1 hour of incubation.

**pH Optimum**—The optimal pH for the enzyme reaction was determined from pH 3 to 7 in 0.1 M Tris-acetate buffer. A single pH optimum around 4.5 to 5.0 was observed, with the highest enzymatic activity located at pH 4.7 (Fig. 10).

Heat Stability—In this study, the purified enzyme was diluted with 0.1% bovine serum albumin in 0.1 M acetate buffer (pH 5.0) or phosphate buffer (pH 7.0) and incubated at various temperatures up to 80°C for 10 min before assay of the activity was carried out at 37°C. As shown in Fig. 11, the enzyme is stable at 55°C for at least 10 min. At 65°C, 67% (at pH 5.0) to 20% (at pH 7) of enzyme activity was lost during 10 min of incubation.

The enzyme is slightly more stable at pH 5 than at 7 with temperatures higher than 55°C.

**Effect of pH on Enzyme Stability**—The diluted enzyme (in 0.1% bovine serum albumin) was incubated at pH 2 to 12 maintained by 0.1 M acetate-phosphate-carbonate buffer for 30 min at 37°C or 24 hours at 4°C. After the incubation, the pH of the enzyme solution was readjusted to pH 5.0 before assaying. As shown in Fig. 12, the enzyme is stable between pH 4 to 11. At pH 3, 30 min at 37°C inactivated 40% of the enzyme while 24 hours at 4°C completely abolished the enzyme activity.

**DISCUSSION**

$\beta$-Glucuronidase has been prepared in a high degree of purity from several sources. Thus, Plapp and Cole (21) have purified the enzyme from bovine liver, 4400-fold (specific activity 190,000 Fishman units/mg of protein), Delvin and Gianetto (23) from rat liver lysosomes, 1090-fold (126,000 units/mg of protein). Stahl and Touster (22) purified it to a much higher purity (8,400-fold, $1.0 \times 10^6$ units/mg of protein) and Ohtsuka and Wakabayashi (24) from rat preputial gland to a specific activity of over $10^5$ units/mg of protein. Recently, purification of this enzyme from rabbit liver to apparent homogeneity has also been reported (25). However, it is the enzyme in the mouse kidney that is receiving most of the attention in the areas of molecular biology, genetics, and biochemistry, and, therefore, it is essential that the pure enzyme be obtained from mouse kidney and characterized as to subunit structure and molecular size.

**Fig. 9.** Effect of substrate concentration on $\beta$-glucuronidase activity. The assay was carried out at pH 5.2 in 0.1 M acetate buffer with phenolphthalein glucuronic acid as substrate. For each substrate concentration, assays were made at 3-min intervals for 15 min to ensure that initial velocities were being measured. The reaction appears to obey normal Michaelis-Menten kinetics and has a $K_m$ value of $1.18 \times 10^{-4} \text{M}$.

**Fig. 10.** Optimal pH for $\beta$-glucuronidase activity. The enzyme activity was measured from pH 3 to 7 in 0.1 M Tris-acetate buffer. Maximum enzyme activity was observed at pH 4.7 in a second experiment.

**Fig. 11.** Heat stability of purified $\beta$-glucuronidase. The enzyme was diluted with 0.1% bovine serum albumin and preincubated at pH 5.0 (■) or 7.0 (△) at temperature from 0°C to 80°C for 10 min before it was assayed at 37°C. At pH 5, the enzyme is stable up to 65°C, while at pH 7, 29% of its activity was lost during 10 min of incubation at this temperature.

**Fig. 12.** Effect of pH on enzyme stability. The purified enzyme (diluted with 0.1% bovine serum albumin) was incubated at pH from 2 to 12 in 0.1 M acetate-phosphate-carbonate buffer for 30 min at 37°C (■) or for 24 hours at 4°C (△). After incubation, the enzyme solution was readjusted to pH 5.0 with 0.4 M acetate buffer before assaying at pH 5.2. The result shows that the enzyme is stable between pH 4 to 11.
In the present study, the hormone-induced kidney became a rich source of \( \beta \)-glucuronidase; about 2.5 times greater than the enzyme level in rat liver. From the induced kidney, we were able to obtain a \( \beta \)-glucuronidase preparation which is apparently homogeneous in terms of sodium dodecyl sulfate gel electrophoresis, gel filtration in Sephadex G-200, and immuno-diffusion. The specific activity of this preparation, 294,000 units/mg of protein, is 3.5 times higher than that obtained by Pettengill and Fishman (20), which relied on organic solvent and ammonium sulfate precipitation as major steps of purification. This also indicates that the enzyme preparation in the isotope incorporation study of Kato et al. (4), which had a maximum specific activity of 76,000 units/mg of protein at the peak tube of the sucrose density gradient centrifugation, contained less than 25% of enzyme protein. This fact would now make their conclusions tentative as purity was assumed.

In the present study, the use of ultrasonication to solubilize the enzyme from the lysosomes and the microsomal membrane and the use of isoelectric focusing for the purification appears to be significant. The ultrasonication solubilized 92% of the enzyme from the tissue homogenate with a 2-fold purification. The isoelectric focusing step achieved a 3-fold purification at the final stage of the procedure. It is possible that this step can be employed at an earlier stage of purification to shorten the entire process and achieve the same degree of purification with higher recovery of the enzyme. This purification procedure now appears to be useful for further studies of intracellular transport of this enzyme, particularly in the use of radioactive incorporation techniques.

There is no experimental evidence reported to indicate that the enzyme induced by androgenic hormones is different from that of normal kidney. In the induced kidney, however, there is an increase in the proportion of the microsomal isoenzyme forms which are converted readily to the lysosomal form (16, 18). In the present experiment, the microsomal forms were converted into the lysosomal form at the ultrasonication step. The resulting purified enzyme, although electrophoretically identical with the lysosomal form (L), originated from all the isoenzyme forms, including those of the microsomes. The ultrasonication step apparently has caused the dissociation of the accessory polypeptide chains from the microsomal isoenzyme components. The final enzyme preparation, although apparently homogenous by criteria presented in this paper, can be further separated into four bands by isoelectric focusing in polyacrylamide gels (18), possibly due to difference in carbohydrate content of the molecules.

\( \beta \)-Glucuronidase is highly stable at elevated temperature and extremes of pH. The \( K_m \) of 1.1 \( \times 10^{-4} \) M obtained in our study is close to the reported values of this enzyme from mouse kidney and from other sources (8, 29–32, 35). Although double pH optima of \( \beta \)-glucuronidase have been reported in a number of publications (22, 36, 37), our result showed a single pH optimum at pH 4.7 which is identical with that reported by Plapp and Cole (21) on the purified bovine liver enzyme.

The properties of the purified enzyme resembled those of the rat liver enzyme reported by Stahl and Touster (22) and of the rabbit liver enzyme reported by D'can (25). From the data reported in the literature and our experimental results, it can be concluded that \( \beta \)-glucuronidase of mouse kidney is a tetrameric structure of 300,000 with subunits of 74,000 in molecular weight. There is no evidence that different types of active subunits exist in a single molecule.

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