The Purification and Properties of β-Galactosidase from Bovine Testes*

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SUMMARY
A β-galactosidase was purified 600-fold from bovine testes by ammonium sulfate precipitation, acetone fractionation, and affinity chromatography on agarose substituted with terminal thio-β-galactopyranosyl residues. The preparation was devoid of protease, a-fucosidase, α-mannosidase, β-glucosidase, β-glucuronidase, and hyaluronidase activities. A wide variety of compounds containing terminal non-reducing galactose residues were hydrolyzed by the enzyme, including proteoglycans, glycoproteins, gangliosides, disaccharides, and nitrophenylgalactosides. Kinetic studies showed that galactose was released at approximately the same maximal rate from several substrates, but that the $K_m$ values differed widely. The enzyme exhibited a high affinity for nitrophenyl galactosides ($K_m$ less than $10^{-4}$ M). The purified β-galactosidase had a pH optimum of 4.3, required sulfhydryl groups for activity, and was neither stimulated nor inhibited by alkali-metal ions or EDTA. A single band of β-galactosidase activity was observed after disc gel electrophoresis at several pH values. At each pH value the enzyme activity corresponded in migration to the major protein band. The β-galactosidase eluted as a single peak from Sephadex G-200 (apparent molecular weight 68,000). The enzyme catalyzed the transfer of galactose from $\beta$-nitrophenyl-β-galactoside to glucose, N-acetylglucosamine, and N-acetylgalactosamine. Galactose, galactal, galactonolactone, and thio-β-galactopyranosides inhibited the enzyme.

Two distinct β-galactosidase activities were shown in bovine liver extracts. One enzyme (or enzymes) (type I) was bound to the affinity column whereas the second enzyme (or enzymes) (type II) passed through the column. While type I β-galactosidase had properties similar to those ascribed to the testicular enzyme, type II β-galactosidase did not hydrolyze lactose or transfer galactose from β-galactosides to glucose.

While β-galactosidases are ubiquitous in animals, plants, and microorganisms (1), the numbers and functions of these enzymes in specific mammalian cell populations are not clearly defined. In humans, β-galactosidase activity occurs in intestinal mucosa (2) and secretions (3) where their role in the digestion of disaccharides has been shown (4). In tissues of patients with Krabbe’s disease (5) and generalized gangliosidosis (6) the lack of specific β-galactosidases is associated with the accumulation of galactose-containing glycolipids. This suggests that these β-galactosidases have a role in the regulation of glycolipid levels in tissues (7). The possibility that galactose-containing glyco-proteins and mucopolysaccharides are similarly catabolized and their metabolism regulated by β-galactosidases has been suggested but not proven (8).

Some mammalian β-galactosidases do not attack high molecular weight β-galactosides. For example, galactose is not removed from desialyzed glycoprotein by β-galactosidase from calf intestinal mucosa (9) or human liver (10). However, rat liver lysosomes release galactose from glycoproteins (11) and crude testicular hyaluronidase preparations can hydrolyze terminal galactose residues from glycoproteins (12) and from proteoglycans (13). These observations prompted the present study, which is concerned with the isolation and characterization of testicular β-galactosidase.

The application of the technique of affinity chromatography to the purification of mammalian β-galactosidases is described.

MATERIALS AND METHODS
Galactose dehydrogenase, galactose oxidase, and hyaluronidase (500 USP units per mg) were obtained from Worthington Biochemical Corp., Freehold, N.J. Hyaluronidase was purified by chromatography on DEAE-cellulose (14) prior to use. Bovine liver β-glucuronidase (type B-10) was obtained from Sigma Chemical Co., St. Louis, Mo. The following compounds were obtained from the sources indicated: Bio-Gel A-15m (50 to 100 mesh) and succinylaminoolalkyl Bio-Gel A-15m (Affinos 202), Bio-Rad Laboratories, Richmond, Calif.; α- and β-nitrophenyl-β-Gal, Pierce Chemical Co., Rockford, Ill.; isopropylthio-β-Gal and γ-galactonolactone, Sigma Chemical Co., St. Louis, Mo.; and p-aminophenylthio-β-Gal and Acocoll, Calbiochem, La

†† These studies were performed under the tenure of a post-doctoral fellowship from the Arthritis Foundation.

* This work was supported in part by Grant AM 10531 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and a grant from the Arthritis Foundation, Michigan Chapter.
treatment of the DEAE-column with progressively higher salt concentrations eluted other protein fractions containing lower ratios of xylose to N-acetylglucosamine.

**Enzyme Assay**

Two assay procedures were utilized to determine β galactosidase activity.

**Assay 1**—The first assay procedure involved measurement of p-nitrophenol or o-nitrophenol as described by Kubz and Lardy (22). Unless otherwise indicated, incubation mixtures contained the following components in total volumes of 0.1 ml: sodium phosphate-citrate buffer, pH 4.3, as prepared by Melville (23), 25 μl; bovine serum albumin, 100 μg; o- or p-nitrophenol-β-Gal, 0.5 μmole; and 1 to 4 units of enzyme. Control tubes contained the same components but lacked either substrate or enzyme. Incubations were conducted for 30 min at 37° and were terminated by the addition of 1 ml of 0.25 M glycine buffer, pH 10. Absorbance was measured in cells with a 1-cm light path at 400 nm for p-nitrophenol and at 420 nm for o-nitrophenol. Enzyme activity was linear with time and protein concentration. The molar extinction coefficient observed with standard p-nitrophenol was 1.75 × 10⁶ and with o-nitrophenol was 4.16 × 10⁵. When required, small quantities of galactose released in the presence of limiting concentrations of nitrophenylgalactosides were determined by a more sensitive assay procedure. A solution (9.9 ml) containing 2.5 ml of citrate-phosphate buffer, pH 4.3, 10 mg of albumin, and an appropriate quantity of nitrophenyl-β-Gal was warmed to 37°, 100 μl of enzyme (1 to 4 units) were added, and the mixture incubated at 37° for 30 min. The reaction was terminated by addition of 2 ml of 2.5 M glycine buffer, pH 10, and the absorbance of liberated nitrophenol was determined in cells with a 1-cm light path.

**Assay 2**—The release of free galactose from galactosides was measured in this procedure. The incubation conditions were those described above for Assay 1 (0.1 ml volume). Following incubation at 37° for 30 min, 0.5 ml of 0.2 M sodium phosphate buffer, pH 7.2, was added and the mixture was heated for 3 min at 100°. After cooling to room temperature, 0.5 ml of a solution containing 0.3 mg of NAD and 10 μg of galactose dehydrogenase was added. The concentration of NADH was determined after 30 min at 340 nm (24). Control incubations contained the same components but lacked galactose dehydrogenase. Solutions of known galactose concentrations served as reference standards.

A unit of enzyme was defined as that amount which released 1 μmole of galactose from p-nitrophenyl-β-D-galactopyranoside in 1 min under the assay conditions described above.

**Purification of Enzyme**

Unless otherwise indicated, all manipulations were performed at 4°.

**Step 1. Crude Extract**—Extraction of β-galactosidase was carried out by a modification of a procedure developed for preparation of hyaluronidase (25). Fresh bovine testes (650 g) obtained from a local slaughterhouse were freed of associated tissues and minced in a precooled meat grinder. The minced tissue was mixed with 615 ml of 0.1 M acetic acid and the pH adjusted to 4.0 by slow dropwise addition of 65 ml of 2.0 N HCl. The homogenate was stirred for 4 hours, centrifuged at 10,000 × g, and the precipitate discarded.

**Step 2. Ammonium Sulfate Precipitation**—The crude supernatant (750 ml) was brought to 20% (NH₄)₂SO₄ saturation by addition of 10.6 g of (NH₄)₂SO₄ for each 100 ml. The mixture

Jolla, Calif. Galactal was obtained from Koch-Light Laboratories, Coinbrook, England, and recrystallized from ethyl acetate. The α and β anomers of p-nitrophenyl-1-Ara were prepared by the method of Feier and Westphal (15) and N-acetylatedosamine from N-benzyl-(3-β-Gal-D-mannosyl)-amine by the method of Kuhn and Kirschenlohr (16). All other chemicals were from commercial sources and were of the highest grade.

The following compounds were generous gifts: desialyzed orosomucoid, Dr. K. Schmid, Boston University; bovine keratan sulfate, Dr. V. Hascall, University of Michigan; [14C]-galactosylglycosylipids (galactosylseramide, lactosylceramide, GM₁ ganglioside, and ceramide lacto-N-tetraose), Dr. S. Basu, University of Notre Dame; O-β-Gal-N-acetylmannosamine, Dr. M. C. Chik, University of Pennsylvania; the 1→3 and 1→6 isomers of N-acetyltosaminosamine, Dr. A. Gauhe, Max-Planck Institute, Heidelberg; and O-β-Gal-(1→4)-N-acetylgalactosamine from Dr. E. Klenk, University of Köln.

**Analytical Procedures**

Protein was measured by the procedure of Lowry et al. (17). Analysis of bound neutral sugars was carried out by gas liquid chromatographic analysis of the corresponding amidotol acetate derivatives (18). The hexosamine content was determined with an amino acid analyzer after prior acid hydrolysis with 4 N HCl for 6 hours.²

Disc gel electrophoresis was performed at pH 4.3, 7.0, and 9.5 in buffers recommended by the instrument manufacturer (Cana-loe, Bethesda, Md.). Proteins were stained with Amido black reagent (19). To detect enzyme on disc gel columns, duplicate gels were run in the presence of sodium thioglycollate (19); one gel was stained and the other gel was cut into 1-mm slices and assayed for enzymatic activity. A correction was applied for shrinkage of the gels during staining.

**Preparation of Proteoglycan “Core” Fractions**

Bovine nasal proteoglycan (4.4 g) (20) was treated for 18 hours with 100,000 units of hyaluronidase in 100 ml of 0.1 M sodium acetate, pH 5.0, containing 2.5% dioxane and 0.05% phenylmethylsulfonylfluoride. After gel filtration on Sephadex G-75 (21), the fractions containing partially degraded proteoglycan were pooled and retreated twice with hyaluronidase to assure maximum elimination of the polysaccharide chain. The final Sephadex eluent, containing proteoglycan degraded to the linkage region, was dialyzed against 20 volumes of distilled water and lyophilized. To remove terminal glucuronic acid residues the powder (1.1 g) was treated with 100,000 units of β-glucuronidase for 24 hours under the conditions described above. The incubation mixture was dialyzed against 0.01 M KCl and applied to a column, 2 × 15 cm, of DEAE-cellulose (chloride form). The column was washed with 100 ml of 0.01 M KCl and eluted with 200 ml of 0.1 M KCl. The latter eluent, devoid of hydrolytic enzymes, contained 5% of the protein-bound xylose applied to the column. The fractions containing protein were pooled, dialyzed against distilled water, and lyophilized. The product (297 mg) gave the following analyses: protein, 48% (by weight); xylose, 46 μmole; galactose, 122 μmole; N-acetylgalactosamine, 62 μmole; and N-acetylglucosamine, 29 μmole. The high ratio of xylose to N-acetylglucosamine suggests that this fraction contains a high proportion of chondroitin sulfate linkage region “stubs” to keratan sulfate chains. Subsequent

² These analyses were kindly performed by Dr. V. Hascall, Department of Oral Biology, University of Michigan Dental School.
was stirred for 1 hour, centrifuged at 10,000 × g, and the precipitate discarded. Ammonium sulfate was added to the supernatant to 75% saturation, 33.7 g for each 100 ml, and after stirring for 3 hours, the precipitate was collected by centrifugation. The supernatant was lyophilized, yielding a powder (5.4 g) containing 110,000 units of β-galactosidase.

When stored at -20°C, the preparation retained full activity for at least 1 year. The concentrate was stable for several weeks when stored at 0°C.

Step 3. Acetone Fractionation—Lyophilized (NH₄)₂SO₄ fraction (1.6 g) was dissolved in 400 ml of distilled water and 125 ml of cold acetone were added. The mixture was stirred for 20 min and a flocculent precipitate was removed by centrifugation. Cold acetone (75 ml) was added to the supernatant and after 20 min with intermittent stirring, the flocculent precipitate was collected by centrifugation and dissolved in 100 ml of distilled water. The acetone preparation was stable for several weeks when stored at 0°C.

Step 4. Affinity Chromatography—Agarose covalently linked to phenylthiogalactopyranoside was prepared by condensation of p-aminophenylthio-β-Gal with succinylaminoalkyl Bio-Gel A-15m as described by Cuatrecasas (26). A column, 1 × 5 cm, of the inhibitor-substituted agarose was washed sequentially with the following solutions prior to use: Tris-HCl buffer, 0.05 M, pH 7.5, 50 ml; water, 50 ml; and McIlvaine buffer, pH 4.3, 50 ml. The acetone fraction from Step 3 (20 ml) was mixed with 10 ml of McIlvaine buffer, pH 4.3, and 10 ml of water. The mixture was passed through the affinity column at 1 ml per hour and the column washed with 80 ml of McIlvaine buffer, pH 4.3, diluted 1 part buffer to 3 parts water. The enzyme was eluted from the column with 20 to 50 ml of 0.05 M Tris-HCl, pH 7.5.

In order to reduce the small amount of contaminating hexosaminidase activity, the affinity chromatography step was repeated. The affinity column eluent containing β-galactosidase activity was adjusted to pH 4.3 with 2 N acetic acid. Albumin was added to bring the protein concentration to 1 mg per ml and the solution applied to a recharged affinity column. In some experiments N-acetylglucosamine was added to a final concentration of 0.1 M. The column was washed and eluted as described above. Enzyme activity was recovered in yields of 75 to 95%. After addition of sodium chloride to a final concentration of 0.15 M, the eluent was concentrated by pressure filtration over an Amicon UM 20E filter. The preparation was stable at -20°C for 6 months when stored in either 0.1% albumin or 30% glycerol.

RESULTS

Testicular β-galactosidase was purified 600-fold as outlined in Table I. The specific activity of the purified preparation ranged between 3000 and 4000 units per mg of protein.

Final purification was achieved on affinity columns containing succinylaminooalkyl Bio-Gel A-15m covalently bound to p-amino-phenylthio-β-galactoside (Fig. 1). Maximum adsorption of the testicular enzyme occurred at pH 4.3 and is consistent with the pH optimum of this enzyme (Fig. 2). In contrast to Escherichia coli β-galactosidase (27), testicular β-galactosidase was found to bind to agarose in the absence of the succinylaminooalkyl ligand or substituted phenylthio-β-Gal. For example, when a 4-ml column of unsubstituted agarose was challenged with 1200 units

**Table I**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume ml</th>
<th>Total protein mg</th>
<th>Total units</th>
<th>Specific activity, units/mg</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>3250</td>
<td>41,300</td>
<td>200,000</td>
<td>4.8</td>
<td>100</td>
</tr>
<tr>
<td>Step 1: Extraction, pH 4.0</td>
<td>750</td>
<td>12,000</td>
<td>171,000</td>
<td>14.2</td>
<td>85</td>
</tr>
<tr>
<td>Step 2: Ammonium sulfate</td>
<td>1200</td>
<td>5,060</td>
<td>119,000</td>
<td>21.4</td>
<td>55</td>
</tr>
<tr>
<td>Step 3: Acetone fractionation</td>
<td>675</td>
<td>983</td>
<td>94,000</td>
<td>96.0</td>
<td>47</td>
</tr>
<tr>
<td>Step 4: Affinity chromatography</td>
<td>125</td>
<td>22.1</td>
<td>74,500</td>
<td>330.0</td>
<td>37</td>
</tr>
</tbody>
</table>

* Purification was based on the extraction of 650 g of bovine tissue.

![Fig. 1. Affinity column chromatography of β-galactosidase from bovine testes.](http://www.jbc.org/)

**Fig. 1.** Affinity column chromatography of β-galactosidase from bovine testes. Acetone fraction (2500 units, pH 4.3) was applied to a column (1 × 5 cm) of phenylthio-β-galactoside-substituted agarose. The column was washed with pH 4.3 buffer and eluted with Tris-HCl buffer, pH 7.5, as described under "Materials and Methods"; 5-ml fractions were collected. ---, protein; ——, β-galactosidase activity.

![Fig. 2. Effect of pH on β-galactosidase activity.](http://www.jbc.org/)

**Fig. 2.** Effect of pH on β-galactosidase activity. Citrate-phosphate buffer described by McIlvaine (23) was used. The substrates were: p-nitrophenyl-β-D-galactopyranoside (Assay 1), ○—○; and lactose (Assay 2), O——O. Incubations contained 2.0 units of testicular galactosidase purified by affinity chromatography.
of \(\beta\)-galactosidase, 35% of the enzyme was bound to the column (Table II). The efficiency of binding of \(\beta\)-galactosidase to un-substituted agarose was reduced by pretreatment of the agarose with either testicular \(\beta\)-galactosidase or galactose oxidase. Extensive treatment with either enzyme failed to eliminate the ability of the support to bind \(\beta\)-galactosidase. When a column of thiogalactoside-substituted agarose was challenged with 2700 units of \(\beta\)-galactosidase, approximately 85% of enzyme was bound to the column. Hence, the phenylthiogalactoside-substituted agarose binds galactosidase more efficiently than unsubstituted agarose.1

While the amount of the \(\beta\)-galactosidase retained on the affinity chromatography column was a function of the amount of enzyme applied to the column, it was also related to the quantity of associated protein. Most efficient binding occurred when less than 10 mg of protein were applied per ml of the phenylthio-\(\beta\)-Gal (Fig. 1). The activity of the enzyme was not changed by substituting K+ for Na+ in the citrate-phosphate buffer, by the presence of 10\(^{-5}\) M EDTA or CaCl\(_2\), or by replacing the buffer in the assay system with 0.2 M sodium acetate, pH 4.3. The enzyme was not active in the presence of \(4 \times 10^{-4}\) M p-hydroxymercureibenzoate, but full activity was restored upon addition of glutathione or cysteine (0.02 M final concentration). Addition of reducing agents was not required during any stage of purification or storage of the enzyme.3

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Purity of \(\beta\)-Galactosidase—The purification procedure removed \(\alpha\)-mannosidase (29), \(\alpha\)-fucosidase (30), \(\beta\)-glucuronidase (31), and hyaluronidase (25) activities present in crude testicular extracts; the purified preparation did not hydrolyze p-nitrophenyl deriva-tives of \(\beta\)-l-arabinopyranoside, \(\alpha\)- or \(\beta\)-l-glucopyranoside, \(\beta\)-xylopyranoside, or \(\alpha\)-galactopyranoside (Assay 1). No proteolytic activity was detected in the purified enzyme preparations after prolonged incubation with Azocoll.

Even the most highly purified \(\beta\)-galactosidase preparations contained hexosaminidase activity. At equivalent substrate concentrations p-nitrophenyl-\(\beta\)-N-acetyl-\(\beta\)-glucosaminide was hydrolyzed 3 times more rapidly than the corresponding \(\beta\)-N-acetyl-\(\beta\)-galactosaminide. No change in the relative activity of the hexosaminidase toward the two substrates was noted at any stage during the purification procedure. Hexosaminidase activity was most prominent in preparations in which sharp separation from galactosidase activity was not achieved during acetone fractionation (Step 3). However, hexosaminidase activity was reduced by sequential affinity chromatography (Table III) and the inclusion of 0.1 M N-acetylglucosamine (final concentration) in the McIlvaine buffer used for the affinity chromatography step. While the ratio of activity of \(\beta\)-hexosaminidase to \(\beta\)-galactosidase changed during purification, the \(\alpha\)-arabinosidase to \(\beta\)-galactosidase ratio remained essentially constant (Table III).

A single major band was detected when the purified \(\beta\)-galacto-sidase was subjected to disc gel electrophoresis at pH 9.5, 7.0, and 4.3 (Fig. 3). Galactosidase activity (Assay 1) was detected in slices of gels run at pH 7.0 and 4.3. The enzyme activity corresponded to the position of the major protein band on stained columns. However, enzyme activity was detected only after prolonged incubation (24 hours). No galactosidase activity was detected in gel columns run at pH 9.5.

### Table II

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Units of galactosidase added</th>
<th>Units of galactosidase bound</th>
<th>Per cent of galactosidase bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Gel A-15m (untreated)</td>
<td>1200</td>
<td>418</td>
<td>35</td>
</tr>
<tr>
<td>Bio-Gel A-15m (galactosidase-treated)</td>
<td>1200</td>
<td>279</td>
<td>23</td>
</tr>
<tr>
<td>Bio-Gel A-15m (galactose oxidase-treated)</td>
<td>1200</td>
<td>159</td>
<td>13</td>
</tr>
<tr>
<td>Bio-Gel A-15m covalently attached to phenylthio-(\beta)-Gal</td>
<td>2700</td>
<td>2240</td>
<td>83</td>
</tr>
</tbody>
</table>

# Visual inspection of the color developed using a coupled galactose oxidase-peroxidase-o-toluidine system (29) indicated a concentration of terminal galactose residues on agarose in the range of 3 to 5 \(\mu\)moles per ml, whereas thiogalactoside-substituted agarose contained 8 to 10 \(\mu\)moles per ml. Absorption of the colored indicator onto the beads prevented accurate estimations.1

### Table III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl-(\beta)-GaldGal</td>
<td>0.007</td>
</tr>
<tr>
<td>p-Nitrophenyl-(\alpha)-Ara</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Enzymes associated with \(\beta\)-galactosidase during sequential affinity chromatography

Incubations were performed as described for Assay 1 ("Materials and Methods") except that \(p\)-nitrophenyl-\(\beta\)-Gal was replaced with the indicated substrate.
The enzyme eluted as a single peak when subjected to gel filtration on Sephadex G-200 (32) and had an estimated molecular weight of 68,000 (Fig. 4).

**Effect of Substrate Concentration**—The effect of varying substrate concentrations on reaction rate when o- and p-nitrophenyl-
β-Gal served as substrates is shown in Fig. 5. The results obtained when lactose, N-acetylactosamine, or desialyzed orosomucoid served as substrate are presented in Fig. 6. The apparent Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) for each substrate were calculated from the Lineweaver-Burk plots and are presented in Table IV. While different β-galactosides had nearly the same $V_{max}$ values, large differences in the $K_m$ values were found. A high affinity for nitrophenylgalactosides ($K_m$ less than $10^{-4}$ M) was exhibited by the testicular enzyme, necessitating development of a highly sensitive assay procedure (described under "Materials and Methods") to measure initial velocities at limiting substrate concentrations. In experiments in which p-nitrophenyl-α-Ara served as substrate, lower $V_{max}$ values were observed.

**Fig. 3.** Disc gel electrophoresis of β-galactosidase. Gel A, pH 4.3, ammonium sulfate fraction (3 units of galactosidase); Gel B, pH 4.3, acetone fraction (9.5 units of galactosidase); Gels C, D, and E each contained 175 units of galactosidase purified by affinity column chromatography and were run at pH 4.3, 7.0, and 9.5, respectively. The gels were stained with Amido black stain. Disc gel electrophoresis was run at pH 7.0 without stacking gel.

**Fig. 4.** Gel filtration of β-galactosidase from bovine testes on Sephadex G-200. The enzyme (250 units) was chromatographed in the presence of 1 mg of bovine albumin to protect the enzyme. The column (1 X 80 cm) was equilibrated and eluted as described by Andrews (32).

**Fig. 5.** Lineweaver-Burk plots for o- and p-nitrophenylgalactosides. Assay 1 was used to determine initial reaction rates. Incubation mixtures in a total volume of 10 ml contained p-nitrophenyl-β-Gal (● — ●) or o-nitrophenyl-β-Gal (■ — ■), and 0.9 unit of purified testicular β-galactosidase. The inset presents results obtained with incubations containing p-nitrophenyl-α-Ara as substrate and 20 units of enzyme.

**Fig. 6.** Lineweaver-Burk plots for desialyzed orosomucoid and disaccharides. Assay 2 was used to determine initial reaction rates. Incubation mixtures contained N-acetylactosamine (● — ●), lactose (■ — ■) or orosomucoid (▲ — ▲), and 1.4 units of β-galactosidase.
TABLE IV  
Michaelis constants and maximal velocity for several substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl-β-Gal</td>
<td>1.00</td>
<td>2.94 x 10^{-2}</td>
</tr>
<tr>
<td>β-Nitrophenyl-β-Gal</td>
<td>0.74</td>
<td>2.81 x 10^{-3}</td>
</tr>
<tr>
<td>p-Nitrophenyl-α-Ara</td>
<td>0.085</td>
<td>1.75</td>
</tr>
</tbody>
</table>
| 4-O-β-Gal-N-acetylglucosamine (N-acetyl-
  glucosamine)                           | 0.98             | 4.4 x 10^{-2}  |
| 4-O-β-Gal-glucose (dactone)              | 0.94             | 1.8 x 10^{-3}  |
| Orosomucoid, desialylated                | 0.98             | 7.7 x 10^{-2}  |

* The compound did not give a Morgan-Elson chromogen by the procedure of Spivak and Roseman (23) and therefore was assumed to be the 1-4 isomer.

and higher $K_m$ were obtained than with the corresponding galactoside (Table IV).

The hydrolytic action of the enzyme with isomers of N-acetylgalactosamine as substrates is shown in Table V. The 1-3 and 1→4 linked isomers were readily hydrolyzed, while the 1→6 linked disaccharide was only slowly hydrolyzed. The nature of the pentultimate sugar also affected the rate of hydrolysis. For example, O-β-Gal-(1→3)-N-acetylgalactosamine was hydrolyzed more rapidly than was O-β-Gal-(1→3)-N-acetylgalactosamine or O-β-Gal-(1→4)-N-acetylmannosamine (Table V).

Enzymatic Hydrolysis of Galactose from Glycoproteins and Acid Mucopolysaccharides—Galactose was removed from desialyzed orosomucoid by testicular β-galactosidase. The initial hydrolysis of galactose from this substrate proceeded rapidly, followed by a decrease in the rate until 87% of the total galactose was released (Fig. 7). The remaining galactose residues may be substituted with fucose residues (9) and therefore would not be attacked by the β-galactosidase.

Crude preparations of bovine testicular hyaluronidase were previously reported to hydrolyze galactose residues from the linkage region of chondroitin 4-sulfate (13). Therefore, the purified β-galactosidase was tested for its ability to hydrolyze galactose from a bovine nasal proteoglycan “core” preparation rich in linkage area galactose and xylose and which contained little keratan sulfate, and (b) a purified bovine nasal keratan sulfate preparation devoid of xylose. As shown in Fig. 7, testicular β-galactosidase cleaved galactose from each of these polymers. Exhaustive treatment with β-galactosidase resulted in the release of 20% of the total galactose from the proteoglycan “core” preparation while only 9.7% of the galactose was released from keratan sulfate. A portion of the galactose content contained in each polymer was resistant to hydrolysis. This may be due to the fact that only terminal galactose residues were removed during the 72-hour incubation period. Since β-galactosidase-treated proteoglycan “core” served as acceptor for [3H]galactose in the presence of UDP-[3H]galactose and embryonic chick brain UDP-galactose; β-xyloside galactosyltransferase (13) it may be assumed that both linkage area galactosyl residues were removed and that terminal nonreducing xylosyl residues were exposed.

**Action of β-Galactosidase on Lipid Substrates**—The ability of the purified testicular β-galactosidase to release galactose residues from glycolipids was investigated (Table VI). All incubation mixtures contained Triton X-100 to ensure substrate solubility. Ceramide lacto-N-tetraose and GM1 ganglioside were hydrolyzed slowly, if at all.

Stimulation of β-Galactosidase Activity by Carbohydrates—While the propensity of many glycosidases, including β-galactosidase, to carry out glycosyl transfer reactions is well known (34), the increased release of nitrophenol from ω- and p-nitrophenyl-β-Gal in the presence of glucose and other carbohydrates was unexpected. This phenomenon was noted with a variety of sugars and glycosides, including α- and β-glucosides, N-acetyl-ω-glucosamine and -galactosamine, α-galactopyranosides, and xylose (Table VII). The enzymatic hydrolysis of nitrophenyl-β-Gal would be expected to yield equimolar amounts of galactose and nitrophenol. However, when glucose was added to incubation mixtures, the liberation of galactose from lactose or p-nitrophenyl-β-Gal was suppressed (Table VII, Experiments II and IV). These results suggest that in the presence of glucose increased hydrolysis of p-nitrophenyl-β-galactoside occurs, and that a portion of the galactosyl residues are transferred to glucose. The increased rates of hydrolysis found in the presence...
of other carbohydrates suggested that they too accept galactose residues.

Transglycosylation to form new glycosides was shown by paper chromatography. For example, when a neutral fraction obtained from incubation mixtures containing p-nitrophenyl-β-D-galactoside was passed through Whatman No. 1 chromatography paper and developed with ethyl acetate-pyridine-water (8:2:1)) three silver-staining spots with mobility of dianexarhides were observed. The spot of weakest intensity co-chromatographed with lactose (&lactose = Gal, glucose, and testicular β-galactosidase was applied to Whatman chromatography paper and developed with ethyl acetate-pyridine-water (8:2:1)).

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The observation that hexosaminidase activity found in testicular extracts was inhibited by galactose (Table VII, Experiment V) may account for the binding of hexosaminidase to the affinity chromatography support containing β-thiogalactoside residues. As expected, the addition of N-acetylglucosamine, a hexosaminidase inhibitor, to the testicular extracts reduced the binding of hexosaminidase to the affinity columns.

### Table VI

**Action of testicular β-galactosidase on glycolipids**

Substrates were dissolved in chloroform-methanol (2:1) containing 1 mg of Triton X-100 and dried in vacuo. Incubations (0.1 ml) contained in addition to substrate and Triton X-100, 25 ml of McIlvaine buffer, pH 4.3, and 5.4 units of testicular β-galactosidase. After incubation, aliquots were subjected to electrophoresis in 0.05 m sodium tetraborate at 77 volts per cm for 25 min and the 14C compounds migrating at the same rate as free galactose were cut out and quantitated.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity</th>
<th>Amount added</th>
<th>[14C]Galactose liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-β-[14C]Gal-proteoglycan &quot;core&quot;</td>
<td>1.3 × 10^4</td>
<td>6.000</td>
<td>90%</td>
</tr>
<tr>
<td>O-β-[14C]Gal-(1→3)-ceramide</td>
<td>1.24 × 10^4</td>
<td>3.250</td>
<td>0%</td>
</tr>
<tr>
<td>O-β-[14C]Gal-(1→4)-Glc-ceramide</td>
<td>2.1 × 10^4</td>
<td>2.750</td>
<td>2%</td>
</tr>
<tr>
<td>O-β-[14C]Gal-(1→3 or →4)-GlcNAc</td>
<td>1.53 × 10^4</td>
<td>7.50</td>
<td>54%</td>
</tr>
<tr>
<td>Glc-ceramideb</td>
<td>1.86 × 10^4</td>
<td>1.300</td>
<td>16%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Substrate</th>
<th>Addition</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1</td>
<td>p-Nitropheny</td>
<td>N</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
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</tr>
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<tr>
<td>N-Acetylgalactosamine</td>
<td>194</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table VII

**Stimulation and inhibition of β-galactosidase by carbohydrates**

Each incubation was conducted in a final volume of 0.1 ml and contained 25 μmol of added carbohydrate and 0.5 μmol of substrate (in incubation mixtures containing lactose, 4 μmol were added). Incubation conditions were those described under "Materials and Methods."
FIG. 8. Glycosyl transfer by testicular β-galactosidase as a function of glucose concentration. The incubation conditions were those described under "Materials and Methods" using o-nitrophenyl-β-Gal as substrate and 6.0 units of β-galactosidase.

Table VIII
Comparison of properties of bovine testicular and bovine liver β-galactosidases

Bovine liver was extracted and processed through Step 2 of the fractionation procedure described for bovine testicular tissue. A total of 1.5 × 10⁶ units of β-galactosidase were obtained from 495 g of liver. The (NH₄)₂SO₄ fraction (1000 units) was mixed with pH 4.3 buffer and passed in a sequential manner through two affinity columns. A portion of the β-galactosidase activity (4.8%) was retained by the first column (type I); the remaining activity was neither bound nor retarded on either affinity column (type II). The characteristics of each bovine β-galactosidase activity are summarized below and are compared to those found for the testicular preparation.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
<td>Type II</td>
</tr>
<tr>
<td><strong>Activity on p-nitrophenyl-β-Gal</strong></td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Plus glucose</td>
<td>1.96</td>
<td>2.09</td>
</tr>
<tr>
<td>Plus lactose</td>
<td>0.24</td>
<td>0.10</td>
</tr>
<tr>
<td>Activity on lactose</td>
<td>0.31</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Optimum pH</strong></td>
<td>4.3</td>
<td>6.9</td>
</tr>
</tbody>
</table>

*Conditions given in Table VII (Assay 1).

when incubated in the presence of glucose nor inhibited when incubated in the presence of galactose.

**DISCUSSION**

Affinity column chromatography has been used for the purification of several microbial glycosidases including _E. coli_ β-galactosidase (27, 35). In the present study bovine testicular β-galactosidase was purified 30-fold by a combination of conventional steps and an additional 35-fold purification was achieved by affinity column chromatography on substituted agarose containing terminal phenylthio-β-Gal residues. Maximum binding of the enzyme to the substituted agarose occurred at its pH optimum, 4.3.

In contrast to the _E. coli_ enzyme, testicular β-galactosidase was bound to unsubstituted agarose. The latter observation suggests that caution should be exercised in the use of affinity columns with an agarose matrix for the purification of mammalian enzymes other than β-galactosidase (or β-galactosidases). The use of this matrix may lead to contamination of the enzyme being purified with β-galactosidase and hexosaminidase.

Bovine testicular β-galactosidase obtained by the purification procedure described in the present study had a broad substrate specificity. Terminal nonreducing galactose residues were hydrolyzed from lactose, N-acetyllactosamine, desialyzed orosomucoid, keratan sulfate, proteoglycan enzymatically degraded to the terminal nonreducing galactose residues contained in the linkage region, and the glycolipids, ganglioside GM₁ and ceramide lacto-N-tetraose. The enzyme hydrolyzed p-nitrophenyl-α-Ara although at a markedly lower rate than the corresponding galactoside. The ability of some preparations to hydrolyze p-nitrophenyl-β-GlcNAc may be due to contamination with heoxosaminidase. Hexosaminidase activity was markedly reduced by inclusion of N-acetylglucosamine in the binding buffer and by repetition of the affinity column purification step.

While only a single species of β-galactosidase was found in extracts of bovine testis, bovine liver extracts contained two distinct β-galactosidase activities. One enzyme (type I) was bound to the inhibitor-substituted agarose and had properties similar to those described for the testis enzyme. A second β-galactosidase activity (type II) did not bind to affinity chromotography columns. Preliminary studies with human liver, coloreum, and skin fibroblasts also indicate a complexity of β-galactosidase activities; some bind to affinity columns and some do not, suggesting that this phenomenon is not unique to bovine liver. Type II enzyme (or enzymes) has a limited substrate specificity and has only been shown to hydrolyze nitrophenyl β-galactosides.

Bovine testicular β-galactosidase catalyzed a transglycosylation reaction in the transfer of galactose residues from nitrophenyl-β-Gal to several carbohydrates. This observation was not unexpected. Wallenfels and Fischer have demonstrated that β-galactosidase preparations from calf intestinal mucosa catalyzed the transfer of galactose from lactose to glucose (94). However, the extent of transglycosylation and the specificity exhibited by the testicular enzyme were unexpected. Glucose, N-acetylglucosamine, and N-acetylgalactosamine appeared to be excellent acceptors while mannose, L-arabinose, and L-fucose were only slightly active. An accompanying publication reports similar acceptor specificity for the transfer of galactosyl residues to nucleotide monosaccharides (36). When nucleotide monosaccharides were utilized as acceptors, nucleotide disaccharides were synthesized in a highly efficient manner.

The absence of β-galactosidase in tissues from patients with GM₁ gangliosidosis is thought to result in the accumulation of ganglioside GM₁ and keratan sulfate in these tissues, presumably

1 Unpublished observations.

2 The peculiar narrow specificity exhibited by the type II β-galactosidase activity in the present study is strikingly similar to that recently reported for human liver β-galactosidase by Meisler (10) who purified human liver β-galactosidase 900-fold by affinity column chromatography on polymerized bovine γ-globulin containing covalently bound p-aminophenylthiogalactoside.
due to lowered catabolism of these galactose-containing compounds (7). Further, the low \( \beta \)-galactosidase content of tissues from patients with mucopolysaccharidoses was thought to result in the accumulation of mucopolysaccharides due to the formation of excess linkage area galactose residues (8). This hypothesis is not easily reconciled with the recent report that “corrective-factors” for specific mucopolysaccharidoses lack \( \beta \)-galactosidase activity (37). In the present study a mammalian glycosidase was shown to hydrolyze galactose residues from protein-chondroitin sulfate degraded to the terminal galactose residue of the neutral linkage region. Enzymatic regulation of the production of linkage region is therefore a possibility. This hypothesis would require that \( \beta \)-galactosidase and the glycosyltransferases involved in the assembly of the galactose portion of the linkage region occur in the same subcellular compartment. Demonstration of the differential subcellular location of \( \beta \)-galactosidase activities should provide evidence bearing upon the anabolic-catabolic functions of these enzymes.

**Acknowledgments**—The skillful technical assistance of Mrs. Miriam Butsch and Miss Eva Stockhorst is gratefully acknowledged.

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