Testosterone Effects on the Induction and Urinary Excretion of Mouse Kidney Glycosphingolipids Associated with Lysosomes*

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Male C57BL/6J mice were shown to excrete in their urine approximately 50 times as much glycolipid as do female mice. These lipids were primarily galabigosylceramides and globotetraglycosylceramides which contained nonhydroxy and hydroxy fatty acids, sphingosine, and phosphashopside as components of their ceramide moieties. The lipids were shown to be associated with multilamellar bodies. Neutral glycosphingolipids from kidney and urine were separated and analyzed by high performance liquid chromatographic techniques. Male kidneys were shown to contain about twice as much neutral glycosphingolipid as female kidneys. The female kidneys were virtually devoid of galactosylceramides and galabigosylceramides. Implantation of testosterone pellets in female mice resulted in a concomitant increase in urinary glycosphingolipids (>100-fold) and in urinary lysosomal enzymes (about 10-fold). In mutant light ear mice, which are defective in excretion of lysosomal enzymes, the urinary excretion of glycolipids is also blocked. Taken together these data suggest that testosterone induces the formation of multilamellar kidney lysosomes which contain a complement of glycosphingolipids and which are normally excreted in the urine.

The proximal tubule cells of mouse kidney respond to testosterone by hypertrophy and by specific induction of several enzymes, including lysosomal glycosidases (1). A direct effect of testosterone on the in vitro rate of synthesis of two lysosomal enzymes, β-glucuronidase (2) and β-galactosidase (3), has been demonstrated. The mechanism of β-glucuronidase induction includes an increase in the concentration of its mRNA (4). Other testosterone-inducible mRNAs in mouse kidney have also been identified (5). After testosterone treatment, a large percentage of the lysosomal enzyme content of the kidney is excreted each day in the urine, and the function of this excretion is not known.

A marked effect of testosterone on the quantity and composition of mouse kidney glycolipids has also been described. In a series of reports which appeared between 1968 and 1971, Gray and associates (7-11) described the kidney glycosphin-

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1 The abbreviations and nomenclature used for glycosphingolipids are those recommended by IUPAC (1976) Lipids 12, 455-468 and are: GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; Gaα1Cer, galabigosylceramide, Galα1 →4)Gal(β1 →1)Cer; Gaα3Cer, globotetraglycosylceramide, Galα1 →4)Gal(β1 →3)Glc(β1 →1)Cer; Gaβ3Cer, globotetraglycosylceramide, Galβ1 →4)Gal(β1 →3)Glc(β1 →1)Cer. HPLC, high performance liquid chromatography, AUPS, absorbance units full scale.
Testosterone Effects on Mouse Kidney Glycosphingolipids

Whole kidneys were taken, weighed, homogenized in methanol, aliquots equivalent to 50 to 100 mg of tissue were extracted with chloroform-methanol, and the glycolipid fraction was isolated, benzoylated, and a portion of the derivatives injected for HPLC analysis. Detection was at 230 nm with 0.16 AUFS sensitivity. Standard peaks are labeled as: A, GlcCer; B, internal standard, IS; C, LacCer; D, GbOse4Cer; E, GbOseoCer. The major components of kidney peaks are labeled as: 3, Glc-Sph-Nfa; 4, Gal-Sph-Nfa; 5, Glc-Phyto-Nfa; 6, Glc-Sph-Hfa; 7, GaOseSph-Nfa; 8, GaOseo-Sph-Hfa; 9, GbOse4-Sph-Nfa; 10, GbOseo-Sph-Hfa; 11, GbOseo-Phyto-Hfa. See Table II for more detailed information on the composition of the peaks.

Urine Collection—Twenty-four- or 48-h urine samples were collected from groups of animals in polycarbonate metabolism cages (Fisher Scientific, Springfield, NJ). For analysis of urinary sediments, urine was collected for 6 h in vessels maintained on ice which contained 2 ml of 0.25 M sucrose in 0.05 M Tris buffer, pH 8.2.

Enzyme Assays—β-Galactosidase and β-hexosaminidase were assayed with p-nitrophenyl-β-D-galactoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide, (Sigma) as previously described (3). Zero time controls were included when urine was assayed.

Analysis and Isolation of Neutral Glycosphingolipids—From 50 to 100 mg of tissue, or urine equivalent to 1 mouse-day, was extracted with chloroform methanol and the glycolipid fraction isolated and...
benzoylated with benzoyl chloride in pyridine for HPLC. The benzoylated glycosphingolipids were separated and quantitated by HPLC on Zipax columns with 230-nm detection with the detector sensitivity set at 0.16 AUFs, as previously described (16). The internal standard N-acetylspermyosine was added to the urine samples collected for the assessment of testosterone induction. For preparative isolation of greater than 1-lg quantities, a Water's Radial Compression Module (RCM) 100 with a Radial-Pak B silica column cartridge was utilized. The gradient employed was 1 to 21% dioxane in hexane in 13 min with a flow rate of 4 ml/min. Fractions were collected by hand with minimum volume tubing connected to the outlet of the detector flow cell. The derivatives were further purified by repeated injection on the Zipax column analytical system and eluted isocratically with dioxane-hexane mixtures, the compositions of which were appropriate for the elution of each particular peak. The purified derivatives were debenzoylated with 0.5 N NaOH in MeOH for 1 h at 37°C and partitioned according to Folch et al. (17). For sugar and fatty acid analysis, the lipids were subjected to methanolysis in anhydrous 0.75 N HCl in methanol as described by Rickert and Sweeley (18). The fatty acid methyl esters were analyzed by gas-liquid chromatography on a 6-foot coiled, 3% OV-1 (on 80 to 100 mesh Supelcoport) column programmed from 190-230°C at 3°C/min with a Hewlett-Packard model 3380A gas chromatograph equipped with a flame ionization detector. Identification of the fatty acids obtained from the total glycolipid fractions were confirmed by electron-impact mass spectrometry with a Finnigan 4000 gas chromatograph-mass spectrometer equipped with a 6115 data system. The methyl glycosides were analyzed as their trimethylsilyl derivatives on the OV-1 column by the procedure described by Rickert and Sweeley (18). For analysis of the long chain bases, the glycolipids were hydrolyzed in 2 N HCl in 82% MeOH as described by Ando and Yu (19). The long chain bases were separated and quantitated by a reverse phase HPLC method as their biphenyl carbonyl derivatives. Details of the long chain base HPLC analysis will be published elsewhere.

Glycosphingolipids-Typical HPLC chromatograms obtained from the analysis of neutral glycosphingolipids from kidney tissues are shown in Fig. 1. The male and female patterns differ in that in the female peaks 4 and 7 are missing and 8 is greatly diminished. For identification of the chromatographic components see below. Quantitative analysis of these components was performed by repeated injection on the Zipax column analytical system and eluted isocratically with dioxane-hexane mixtures, the compositions of which were appropriate for the elution of each particular peak. The purified derivatives were debenzoylated with 0.5 N NaOH in MeOH for 1 h at 37°C and partitioned according to Folch et al. (17). For sugar and fatty acid analysis, the lipids were subjected to methanolysis in anhydrous 0.75 N HCl in methanol as described by Rickert and Sweeley (18). The fatty acid methyl esters were analyzed by gas-liquid chromatography on a 6-foot coiled, 3% OV-1 (on 80 to 100 mesh Supelcoport) column programmed from 190-230°C at 3°C/min with a Hewlett-Packard model 3380A gas chromatograph equipped with a flame ionization detector. Identification of the fatty acids obtained from the total glycolipid fractions were confirmed by electron-impact mass spectrometry with a Finnigan 4000 gas chromatograph-mass spectrometer equipped with a 6115 data system. The methyl glycosides were analyzed as their trimethylsilyl derivatives on the OV-1 column by the procedure described by Rickert and Sweeley (18). For analysis of the long chain bases, the glycolipids were hydrolyzed in 2 N HCl in 82% MeOH as described by Ando and Yu (19). The long chain bases were separated and quantitated by a reverse phase HPLC method as their biphenyl carbonyl derivatives. Details of the long chain base HPLC analysis will be published elsewhere.

**RESULTS**

**HPLC Analysis of Male and Female C57BL/6J Kidney Glycosphingolipids**—Typical HPLC chromatograms obtained from the analysis of neutral glycosphingolipids from kidney tissues are shown in Fig. 1. The male and female patterns differ in that in the female peaks 4 and 7 are missing and 8 is greatly diminished. For identification of the chromatographic components see below. Quantitative analysis of these components was performed by repeated injection on the Zipax column analytical system and eluted isocratically with dioxane-hexane mixtures, the compositions of which were appropriate for the elution of each particular peak. The purified derivatives were debenzoylated with 0.5 N NaOH in MeOH for 1 h at 37°C and partitioned according to Folch et al. (17). For sugar and fatty acid analysis, the lipids were subjected to methanolysis in anhydrous 0.75 N HCl in methanol as described by Rickert and Sweeley (18). The fatty acid methyl esters were analyzed by gas-liquid chromatography on a 6-foot coiled, 3% OV-1 (on 80 to 100 mesh Supelcoport) column programmed from 190-230°C at 3°C/min with a Hewlett-Packard model 3380A gas chromatograph equipped with a flame ionization detector. Identification of the fatty acids obtained from the total glycolipid fractions were confirmed by electron-impact mass spectrometry with a Finnigan 4000 gas chromatograph-mass spectrometer equipped with a 6115 data system. The methyl glycosides were analyzed as their trimethylsilyl derivatives on the OV-1 column by the procedure described by Rickert and Sweeley (18). For analysis of the long chain bases, the glycolipids were hydrolyzed in 2 N HCl in 82% MeOH as described by Ando and Yu (19). The long chain bases were separated and quantitated by a reverse phase HPLC method as their biphenyl carbonyl derivatives. Details of the long chain base HPLC analysis will be published elsewhere.

Electron Microscopy—The 18,000 × g pellet from male urine was fixed in buffered 2.5% glutaraldehyde and 1% osmium tetroxide and postfixed in buffered 2.5% glutaraldehyde and 1% osmium tetroxide. The specimens were then washed in several changes of Millonig's phosphate buffer and dehydrated in an increasing series of ethyl alcohol solutions. The specimens were then infiltrated with a mixture of ethyl alcohol and Epon 812 polymer and embedded in Epon 812 polymer. Ultrathin sections were prepared with a diamond knife on a Porter-Blum MT2 microtome. The sections were stained with uranyl acetate and lead citrate and examine under a Hitachi HU12 electron microscope at an accelerating voltage of 80 kV. The electron micrographs were recorded on Kodak SO301 film at various magnifications.

**Isolation and Analysis of C57BL/6J Kidney Glycosphingolipids**—Fifty male and 40 female kidneys were extracted and the glycosphingolipid fractions prepared. These fractions were benzoylated and the derivatives separated by HPLC as described above, debenzoylated with mild alkali, and analyzed for fatty acids, carbohydrates, and long chain bases. An HPLC chromatogram of longchain bases from total male kidney glycosphingolipids is presented in Fig. 2 as an example. The compositions of the individual components resolved by HPLC are summarized in Table 1. Clearly, the chromatographic resolution of these glycolipid derivatives depends, in this normal phase adsorption mode, not only on the carbohydrate moieties but also on the nature of the fatty acids and long chain bases present. The ceramides consist primarily of C16, C18, C22, C24, nonhydroxy and hydroxy fatty acids along with C18 sphingosine and phytosphingosine. In general, the species which contain hydroxy fatty acids are resolved from those containing nonhydroxy fatty acids, and in the case of the monohexosylceramides, the species containing phytosphingosine and nonhydroxy fatty acids (peak 5) elute before those containing hydroxy fatty acids. In the case of the more complex glycolipids, the hydroxy fatty acid- and phytosphingosine-containing components elute together and are to a large extent resolved from the components which contain nonhydroxy fatty acids and sphingosine. Further characteri-
Glycosphingolipid fractions were isolated from male (M) and female (F) kidneys, benzoylated, and the chromatographic components 3 through 12 (see Fig. 1) isolated by HPLC procedures. Each fraction was debenzoylated and fatty acid, monosaccharide, and long chain base components were analyzed (see "Materials and Methods"). The Glc/Gal/GalNAc ratios were calculated from the responses obtained from standard GlcOse4Cer. The per cent nonhydroxy fatty acids and hydroxy fatty acids (%Nfa and Hfa) were calculated from those individual components which represented more than 5% of the total fatty acid peak area. In all instances, C16, C18, C22, and C24 Nfa and/or Hfa constituted the major fatty acid components. The per cent sphingosine (Sph) or phytosphingosine (Phyto) data were calculated from HPLC analyses as shown in Fig. 2.

<table>
<thead>
<tr>
<th>HPLC peak no.</th>
<th>Glc/Gal/ GalNAc ratio</th>
<th>Per cent Nfa or Hfa*</th>
<th>Per cent sphingosine or phytosphingosine</th>
<th>Abbrev. for major component</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.0:0:0</td>
<td>100 Nfa</td>
<td>100 Sph*</td>
<td>Glc-Sph-Nfa</td>
</tr>
<tr>
<td>4</td>
<td>1.0:2:0</td>
<td>100 Nfa</td>
<td>100 Sph</td>
<td>Gal-Sph-Nfa</td>
</tr>
<tr>
<td>5</td>
<td>1.0:0:0</td>
<td>100 Nfa</td>
<td>100 Phyto</td>
<td>Glc-Phyto-Nfa</td>
</tr>
<tr>
<td>6</td>
<td>1.0:0:0</td>
<td>90 Nfa; 70 Sph;</td>
<td>Glc-Sph-Hfa;</td>
<td>Gal-Sph-Nfa</td>
</tr>
<tr>
<td>7</td>
<td>1.0:0:0</td>
<td>76 Nfa; 72 Sph;</td>
<td>Glc-Phyto-Hfa;</td>
<td>Gal-Sph-Nfa</td>
</tr>
<tr>
<td>8</td>
<td>0:2:0:0</td>
<td>100 Nfa</td>
<td>27 Sph</td>
<td>Gal-Sph-Hfa</td>
</tr>
<tr>
<td>9</td>
<td>0:2:0:0</td>
<td>100 Nfa</td>
<td>14 Phyto</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>10</td>
<td>0:2:0:0</td>
<td>67 Nfa; 53 Phyto;</td>
<td>GbOse,-Sph-Hfa;</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>11</td>
<td>0:1:0:0</td>
<td>74 Nfa; 70 Sph;</td>
<td>GbOse,-Sph-Hfa;</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>12</td>
<td>0:1:0:0</td>
<td>76 Nfa; 72 Sph;</td>
<td>GbOse,-Sph-Hfa;</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>13</td>
<td>0:1:0:0</td>
<td>34 Nfa; 27 Sph;</td>
<td>GbOse,-Sph-Hfa;</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>14</td>
<td>0:1:0:0</td>
<td>100 Nfa</td>
<td>27 Sph</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>15</td>
<td>0:1:0:0</td>
<td>100 Nfa</td>
<td>14 Phyto</td>
<td>GbOse,-Phyto-Nfa</td>
</tr>
<tr>
<td>16</td>
<td>0:1:0:0</td>
<td>69 Nfa; 100 Phyto;</td>
<td>GbOse,-Phyto-Hfa;</td>
<td>GbOse,-Phyto-Nfa</td>
</tr>
<tr>
<td>17</td>
<td>0:1:0:0</td>
<td>31 Nfa; 60 Phyto;</td>
<td>GbOse,-Phyto-Hfa;</td>
<td>GbOse,-Phyto-Nfa</td>
</tr>
<tr>
<td>18</td>
<td>0:1:0:0</td>
<td>44 Nfa; 40 Phyto;</td>
<td>GbOse,-Phyto-Hfa;</td>
<td>GbOse,-Phyto-Nfa</td>
</tr>
<tr>
<td>19</td>
<td>1.0:0:0</td>
<td>100 Nfa</td>
<td>70 Sph</td>
<td>GbOse,-Sph-Nfa</td>
</tr>
<tr>
<td>20</td>
<td>1.0:0:0</td>
<td>80 Nfa; 76 Phyto;</td>
<td>GbOse,-Phyto-Nfa;</td>
<td>GbOse,-Phyto-Nfa</td>
</tr>
<tr>
<td>21</td>
<td>1.0:0:0</td>
<td>20 Hfa; 24 Sph;</td>
<td>GbOse,-Sph-Hfa;</td>
<td>GbOse,-Sph-Hfa</td>
</tr>
</tbody>
</table>

* Nfa and Hfa, refer to nonhydroxy and hydroxy fatty acid, respectively.  
*Sph and Phyto, refer to C18 sphingosine and C18 phytosphingosine, respectively.

TABLE II

Composition of neutral glycosphingolipids from male and female mouse kidneys

Glycosphingolipid fractions were isolated from male (M) and female (F) kidneys, benzoylated, and the chromatographic components 3 through 12 (see Fig. 1) isolated by HPLC procedures. Each fraction was debenzoylated and fatty acid, monosaccharide, and long chain base components were analyzed (see "Materials and Methods"). The Glc/Gal/GalNAc ratios were calculated from the responses obtained from standard GlcOse4Cer. The per cent nonhydroxy fatty acids and hydroxy fatty acids (%Nfa and Hfa) were calculated from those individual components which represented more than 5% of the total fatty acid peak area. In all instances, C16, C18, C22, and C24 Nfa and/or Hfa constituted the major fatty acid components. The per cent sphingosine (Sph) or phytosphingosine (Phyto) data were calculated from HPLC analyses as shown in Fig. 2.

The female kidney is virtually devoid of GalCer and those components of GaOse4Cer containing non-hydroxy fatty acids and sphingosine. The quantities of female components 9, 11, and 12 obtained were insufficient for analysis, but their chromatographic behavior appears identical with the male components. Male peaks 11 and 12 appear to consist primarily of GhOse4Cer but the consistently low values obtained for N-acetylglactosamine may indicate the presence of additional unidentified components. No N-acetylglucosamine was detected in these two peaks.

Thus, the differences in kidney glycolipids of male and female C57BL/6J mice first noted by Coles and Gray (8) has been analyzed in greater detail by HPLC as shown in Fig. 1 and Table I. Most notably, the females lack GalCer (peak 4) and much of the GaOse4Cer (peaks 7 and 8). Lactosylceramide appears to be a component of female peak 8 and is perhaps a

Fig. 3. HPLC chromatograms of male and female C57BL/6J urine glycosphingolipids. Urine aliquots equivalent to 3 mouse-days and 0.1 mouse-day were taken from female and male urine samples, respectively, and processed for HPLC analysis. One-fifth of each sample was injected on the column. Peak identification as in Fig. 1.

TABLE III

Glycosphingolipid content of C57BL/6J mouse urine

Two mice were placed in each plastic metabolism cage and urine was collected over a 48-h period. The glycosphingolipids were isolated from the total urine sample and analyzed by HPLC (see Fig. 3). Urines from four male pairs and three female pairs were collected and analyzed.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.92 (0.82)</td>
<td>0.31 (0.013)</td>
</tr>
<tr>
<td>7</td>
<td>1.57 (0.90)</td>
<td>n.d.*</td>
</tr>
<tr>
<td>8</td>
<td>3.53 (2.72)</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>2.91 (2.05)</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>9.11 (3.90)</td>
<td>0.045 (0.016)</td>
</tr>
<tr>
<td>Total</td>
<td>18.0 (11.7)</td>
<td>0.345 (0.038)</td>
</tr>
</tbody>
</table>

* n.d., not detectable.

Fig. 4. Total neutral glycosphingolipid and lysosomal enzyme levels in female mouse urine following testosterone implantation. The samples were frozen and thawed before assay of the lysosomal enzymes. β-Hexosaminidase values between days 14 and 22 were not obtained. Females without testosterone excrete 0.35 nmol glycolipid/mouse/day (see Table III).
Testosterone Effects on Mouse Kidney Glycosphingolipids

FIG. 5. HPLC chromatograms of urine neutral glycosphingolipids obtained from female mice with subcutaneously implanted 50-mg testosterone pellets. A constant amount of internal standard (I.S.) was added to each sample. Inserts are data from different post-implantation days and are labeled: A, 1 day; B, 5 days; C, 6 days; D, 15 days. See Fig. 1 for peak identification.

trace component in the male. The male kidney contains almost twice as much total neutral glycosphingolipid as does the female and this difference is reflected in all of the individual components except the glucosylceramides (peaks 3 and 5) and the nonhydroxy fatty acid GbOse4Cer (peak 11).

HPLC Analysis of C57BL/6J Urinary Glycosphingolipids—Urine was collected from male and female mice in plastic metabolism cages for 24-h periods. Total urine glycolipids were analyzed and the results expressed as nanomoles of glycolipid per mouse per day. These data are presented in Fig. 3 and Table III. Male mice excrete about 50-fold more glycolipids than females. The HPLC pattern of urinary glycolipids is distinct from that of total kidney glycolipids. In the urine, mono- and tetraglycosylceramides are practically absent.

Testosterone Induction of Urinary Lysosomal Enzymes and Glycosphingolipids in Adult Female Mice—Four female mice were placed in a metabolic cage and urine was collected daily for 7 days. On day 8, testosterone pellets were implanted in each mouse and urine was collected each day for another 28 days. On day 29, the mice were killed and the kidneys removed for analysis of the lysosomal enzyme activities and neutral glycosphingolipids. Each urine sample was analyzed for β-galactosidase, β-hexosaminidase, and glycolipids. The time course of the increase in urinary content of glycolipids and of lysosomal enzymes is indicated in Fig. 4. Both glycolipids and lysosomal enzymes increased dramatically in the period between 4 and 12 days after implantation of testosterone pellets. Enzyme activities in the urine then remained elevated at values roughly 10-fold greater than uninduced females, and total glycolipid concentration remained elevated at more than 100 times the uninduced values.

The time course of appearance of four individual glycolipids (peaks 7, 8, 9, and 10) is presented in Figs. 5 and 6. The GaOse4Cers (peaks 7 and 8) exhibit a different time course from that of the GbOse4Cers (peaks 9 and 10). GaOse4Cers increase dramatically between days 3 to 6 of induction (Fig. 6). At 5 and 6 days after implantation, they comprise a significant proportion of the total glycolipids (Fig. 5, B and C). After day 6, the excretion of the GaOse4Cers is reduced in quantity. In contrast, the amount of GbOse4Cers in the urine continues to increase after day 6, reaching induced values which are several hundred times greater than the uninduced values. As a result of this difference in time course, by day 15 of induction the GaOse4Cers are again minor components (Fig. 5D), as they are in uninduced urine (Fig. 3), while the GbOse4Cers now constitute more than 90% of the total glycolipid. The urine of fully induced females contains a higher proportion of GbOse4Cers (90%) than the urine of male mice (66%) or of uninduced females (1.3%).
Testosterone Effects on Mouse Kidney Glycosphingolipids

Differences in the method of administration and length of exposure of the female mice to testosterone. At 5 or 6 days post-implantation of testosterone, we also observed a large effect on dihexosylceramides.

Analysis of Glycolipids in Urinary Lysosomal Bodies—Normal male urine was collected on ice in 0.25 M buffered

Glycolipid Composition of Testosterone-induced Female Kidney—The composition of kidney glycolipids in fully induced females, on day 29, is indicated by the HPLC pattern in Fig. 7. The pattern resembles the male pattern, with a few notable exceptions. Peak 7, a “male-specific” component, is induced but does not approach levels observed in the male. Peak 10 is increased to higher levels than in the male. In addition, an entirely new component, found in neither male nor uninduced female, is present after induction (peak 8a).

These observations differ from those of Gray (11) who reported an increase in dihexosylceramides but not in trihexosylceramides after daily subcutaneous injection of 0.1 mg of testosterone for 2 weeks. This discrepancy may result from

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**Table IV**

**Glycosphingolipid content of male mouse urine sediments**

<table>
<thead>
<tr>
<th>HPLC peak no.</th>
<th>800 × g pellet</th>
<th>18,000 × g pellet</th>
<th>18,000 × g supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol/ml urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Total urine</td>
<td>15%</td>
<td>68%*</td>
<td>16%</td>
</tr>
</tbody>
</table>

* In a separate experiment, the per cent of total urine glycosphingolipid found in the 18,000 × g pellet was 59%.
sucrose as described under "Materials and Methods." Cells and cellular debris were removed by centrifugation at 800 × g and the supernatant was centrifuged at 18,000 × g. An electron micrograph of the 18,000 × g pellet is shown in Fig. 8. The multilamellar bodies seen closely resemble those reported by Koenig et al. (14). These multilamellar bodies were lysed by freezing and thawing and their membranes collected by centrifugation at 100,000 × g. All pellets and supernatants obtained from the urine were analyzed for neutral glycolipids.

These data are presented in Table IV. Before lysis, the 18,000 × g pellet contained the major portion of the total urine glycolipid; after lysis much of this glycolipid was associated with the membrane fraction.

**Defective Glycolipid Secretion in Mutant le/le Mice—** Mice homozygous for the le mutation are defective in urinary secretion of lysosomal enzymes (3, 21). As a result of this defect, the concentrations of glycosidases and cathepsin C are elevated in the kidneys of male animals. If the testosterone-inducible glycolipids described above are associated with lysosomes we would predict that the genetic lesion in male le/le mice would alter glycolipid concentrations in kidney and urine in the same direction as its effects on lysosomal enzymes.

To test this prediction, glycolipids from kidney and urine of homozygous le/le males were analyzed. As anticipated, the glycolipid concentration in mutant urine was significantly reduced in comparison with that of the C57BL/6J controls (Fig. 9, bottom). In addition, there was a marked accumulation of glycolipids in the mutant kidneys (Fig. 9, top). Qualitatively, the glycolipid pattern of the mutant kidney was similar to that of C57BL/6J male urine (see Figs. 10 and 3). It thus appears that the mutation which reduces the rate of secretion of lysosomal enzymes also prevents the secretion of these glycolipids, causing their accumulation in the kidney. This result supports the hypothesis that the testosterone-induced glycolipids are associated with the lysosomes of mouse kidney.

**DISCUSSION**

Adams and Gray (7) isolated kidney glycolipids from C57BL mice and determined the structures of the carbohydrate moieties by methylation analysis. The monohexosylceramides were reported to consist of 67% GlcCer and 33% GalCer, the di-
hexosylceramide as Gal(1 → 4)Gal(1 → 1)Cer (95%), triglycosylceramides as 100% Gal(1 → 4)Gal(1 → 4)Glc(1 → 1)Cer and the tetracygosylceramides as 100% GalNAc(1 → 3)Gal(1 → 4)Gal(1 → 4)Glc(1 → 1)Cer. No data relating to the anomeric configurations were reported. Subsequent complete structural analyses of which the carbohydrate moieties from glycolipids of other species (22) suggest that these mouse glycolipids probably correspond to gala- and globoglycosylceramides. Our lipids of other species (22) suggest that these mouse glycolipids correspond to galactosyl and globosylceramides. Our analysis of the carbohydrate components of the HPLC separable species (see Table II) are consistent with the conclusion that these mouse kidney glycolipids consist of GlcCer, GalCer, LacCer, GaOseCer, GbOseCer, and GbOse2Cer.

The chromatographic system utilized in these studies provides separation based not only on the carbohydrate moieties but also on the nature of the fatty acids and long chain bases present. Although incomplete resolution of molecular species were obtained and many minor fatty acid and long chain bases are present, the major components in the HPLC peaks (summarized in Table II) indicate that each carbohydrate moiety is associated with a variety of ceramides which contain primarily sphingosine, phytosphingosine, nonhydroxy and hydroxy fatty acids.

The report by Koenig et al. (14) concerning the excretion of lysosomal enzymes in the urine of mice suggested to us that the lysosomal enzymes and glycolipids might both reside in those multi-lamellar bodies which are greatly elevated in the urine by testosterone treatment. We therefore compared the neutral glycolipid content of male and female urine, which were found to differ greatly (see Fig. 3 and Table III). Males excrete roughly 50 times as much glycolipid as do females. It is also noteworthy that the male urinary glycolipid composition is distinct from that of the whole kidney, and that approximately 10% of the total male kidney glycolipid is excreted each day.

In order to examine the temporal relationship between the testosterone-induced excretion of lysosomal enzymes and of glycolipids, the levels of two marker lysosomal enzymes and of glycolipids in the urine were examined daily during the course of induction in female mice. The results in Figs. 4 and 5 demonstrate roughly coordinated increases in enzymes and glycolipids between days 3 to 12, followed by maintenance of elevated plateau levels. The increase in the enzyme activities and in glycolipids are first detectable on day 3 of induction. The GaOseCers rise initially and then fall, while the GbOseCers increase to a much greater extent and do not decrease. Hydroxy fatty acid GbOse2Cer (peak 10) accounts for the bulk of the increase. If urinary enzymes and glycolipids are produced by the excretion of lysosomal bodies, then the glycolipid composition of those lysosomes must be changing during the course of the induction.

To examine the physical relationship between the glycolipids and the urinary organelles, male urine was fractionated by differential centrifugation. The 18,000 × g pellet was subfractionated by freeze/thaw and centrifugation at 100,000 × g. Glycolipids appear to be associated with the sedimentable membrane of the multilamellar bodies (Table IV).

The urinary glycosphingolipids are not degraded during a 48-h period at room temperature and are not sedimentable at 18,000 × g unless this urine is collected on ice.3 The di- and triglycosylceramides (peaks 7 through 10) which terminate in α-galactosyl residues are substrates for lysosomal α-galactosidase in vitro. This α-galactosidase is among the enzymes elevated in male urine.4 The resistance of the urinary glycosphingolipids to urinary α-galactosidase may be explained by the relative lability of this enzyme or by the inaccessible state of the glycosphingolipids within the membrane.

The sexual dimorphism of glycolipid concentration and composition in kidney and urine, and the inductive effects of testosterone in female mice, parallel the known effects of androgens on lysosomal enzymes. Furthermore, the glycolipids are associated with the multilamellar lysosomal bodies which appear in kidneys and urine of testosterone-treated female mice. These observations lead to the hypothesis that testosterone induces the de novo formation of complete lysosomes in the proximal tubule cells of the kidney. Such lysosomes would contain a full complement of lysosomal enzymes as well as a high content of neutral glycosphingolipids in their membranes. A high content of glycolipids has been reported for tritosomes membranes isolated from liver (23, 24). The testosterone-induced lysosomes are postulated to develop into the multilamellar lysosomal bodies which are normally excreted into the urine by an exocytotic process as proposed by Koenig et al. (13, 14). Support for this concept is also provided by the lysosomal enzyme and glycolipid abnormalities in the "light ear" mutant mouse (3, 21). In male le/le kidneys, β-galactosidase is elevated approximately 4-fold (3) and the glycolipids are elevated 10-fold relative to males of strain C57BL/6J. The elevated kidney β-galactosidase in these mutants has been shown to result from the defective excretion of this enzyme; likewise, the excretion of urinary glycolipids by male le/le mice is only 10% as great as in C57BL/6J mice. It thus appears that the mutant mice have a block in the normal excretion of multilamellar bodies, which is reflected in abnormal enzyme and glycolipid levels in kidney and urine.

Three lines of evidence indicate that GaOseCer and GbOseCer are glycolipid constituents of kidney lysosomal membranes: 1) they are the major glycolipids in urine and in the multilamellar bodies isolated from urine; 2) their concentrations are elevated in testosterone-treated females which are producing increased quantities of lysosomal enzymes; and 3) they accumulate specifically in le/le mice which are defective in lysosome excretion. Preliminary data on the glycolipid composition of partially purified kidney lysosomes supports this interpretation.

The data reported here emphasize the fact that relatively large quantities of specific glycolipids can be associated with cytoplasmic lysosomal bodies. Glycolipid changes in cells, tissues, or organs as a function of variables such as age, sex, cell density, or cell type, should not be interpreted as differences in plasma membrane composition without direct evidence regarding the subcellular distribution of the glycolipids involved. The nonspecific increases of glycosphingolipids in several lysosomal storage disorders (25, 26) may reflect in part the accumulation of lysosomal membranes associated with the storage process. This work demonstrates that exocytotic processes, in addition to synthesis and degradation, can have a major influence on tissue levels of glycosphingolipids.

REFERENCES

Testosterone Effects on Mouse Kidney Glycosphingolipids