Biosynthesis of neutral glycosphingolipids in kidney slices from male and female mice[1]

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Abstract Previous reports from our laboratory (1981. *J. Biol. Chem.* **256:** 13112–13120 and 1983. *Endocrinology.* **113:** 251–258) showed the absence of Nfa-GalCer and Nfa-GaOse$_2$Cer in kidneys of several strains of female mice. These lipids are always present in male kidneys and several other glycolipids are also elevated in males. To test whether this phenomenon is due to lowered biosynthesis in females, glycosphingolipid formation was assessed in kidney slices with [^{3}H]galactose as precursor. The glycolipids were extracted after various incubation periods (from 30 min to 90 min) and individual glycolipids were separated and quantitated by high performance liquid chromatography and radioactivity was determined. The rate of formation of hydroxy fatty acid-containing galactosylceramide was the same in both sexes. The glycolipids which were low or not detectable in female kidney, Nfa-GalCer, Nfa-GaOse$_2$Cer and Hfa-GaOse$_2$Cer were rapidly labeled in the male kidney slices. These results suggest that nonhydroxy fatty acid-containing ceramide:UDP-Gal galactosyltransferase and hydroxy fatty acid-containing galactosylceramide:UDP-Gal galactosyltransferase have elevated activities in males. While the glucosylceramides are labeled at the same rates in both sexes, lactosylceramide appears to be labeled at higher rates in the male tissue. This suggests that glucosylceramide:UDP-Gal galactosyltransferase also has elevated activity in males. In addition, these data show that monohexosylceramides with different ceramide compositions are labeled at different rates. – **McCluer, R. H., and S. K. Gross.** Biosynthesis of neutral glycosphingolipids in kidney slices from male and female mice. *J. Lipid Res.* 1985. **26:** 593–599.

Supplementary key words kidney • cerebrosides • galabiglycosylceramide • globoside • testosterone • tissue slices • HPLC • isotope incorporation

The androgen inducibility of β-glucuronidase in mouse kidney proximal tubule cells was first shown by Fishman and Farmeland in 1953 (1) and has served as a model for the examination of the influence of steroid hormones ever since (2). A number of other lysosomal enzymes are also induced and because kidney cells actively secrete lysosomal enzymes into urine, the rate of enzyme loss by secretion increases markedly during induction (3). The induction is confined to proximal tubule cells which undergo hypertrophy but little hyperplasia. Electron microscopy has shown that near the brush border of induced cells there is a concentration of enlarged lysosomes filled with myelin-like figures and the same membranous material is present in the lumen of the proximal tubule and is presumably related to the active secretion of lysosomes. We have previously provided evidence that, in several inbred strains of mice, testosterone induces the biosynthesis of specific di- and triglycosylceramides coordinately with the induction of lysosomal enzymes and that these glycolipids are components of the multilamellar organelles that are normally excreted in the urine (4, 5). The active biosynthesis of glycosphingolipids in mouse kidney and the androgen effects specifically on proximal tubule cells make this tissue suited for studies on the biosynthesis of glycosphingolipids and allow androgen effects on glycolipid content and metabolism to be assigned to a single cell class.

Early in vivo studies on the biosynthesis of glycosphingolipids (6) demonstrated that labeled glycolipids could be isolated but precursor-product relationships were not demonstrable. The difficulties in interpreting such studies are considerable and more recent studies have utilized cell culture systems. Miller-Podraza and Fishman (7) were able to show in oligodendroglioma cells (C6) that monoglycosylceramide was labeled with [^{3}H]galactose and

Abbreviations: 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; GaOse$_2$Cer, galabiglycosylceramide, Gal(α1-4)Gal(β1-1)Cer; GbOse$_3$Cer, globotriglycosylceramide Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; GbOse$_4$Cer, globotetraglycosylceramide, GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer. HPLC, high performance liquid chromatography; AUFS, absorbance units full scale; GSL, glycosphingolipids.

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during a chase period the rate of disappearance of label was greatest while labeling of the more complex glycosphingolipids continued. However, no evidence of precursor-product relationship between other glycolipids was obtained over a 3-day period. The difficulty in finding precursor-product relationships between glycosphingolipids, which are presumably synthesized stepwise with sugar nucleotides, prompted Roseman (8) and Caputto et al. (9) to suggest an enzyme-complex model for the biosynthesis of gangliosides. According to this model, gangliosides originate at specific sites from a limited pool of transient intermediates and once end products are formed they pass to another site from which they are not available as intermediate substrates. Such a mechanism may also apply to neutral glycosphingolipids.

A large number of in vitro studies have demonstrated glycolipid glycosyltransferase activities with nucleotide sugars as donors (10). The issue of transferase specificities and the number of different enzymes involved in biosynthetic pathways for glycolipids has not been established, primarily because these enzymes are membrane-bound and only a few have been solubilized and purified. Even after purification of the solubilized or detergent-treated transferases, it is difficult to determine whether their specificities and properties are different from those in intact membranes. In addition, the influence of the ceramide moiety on the interaction of glycosphingolipids with glycosyltransferases has received little attention. The activity of Hfa-ceramide:UDP-Gal galactosyltransferase has been shown to be three times higher than that of Nfa-ceramide:UDP-Gal galactosyltransferase in mouse brain (11). It is not certain whether these activities are different enzymes or whether the affinity of a single enzyme for Nfa and Hfa ceramide is different. Similar differences in the galactosyltransferase activity with the different ceramides has been demonstrated in mouse kidney microsomes (11, 12).

In the present study we have used kidney slices from male and female mice to measure the rate of incorporation of radiolabeled galactose into mono- and diglycosylceramides that are separated by HPLC according to their carbohydrate and ceramide composition. These studies have provided information about the metabolic distinction of Hfa- and Nfa-containing glycosphingolipids in situ as well as the enzymatic steps that are induced by testosterone and located in the proximal tubule cells.

MATERIALS AND METHODS

Animals

C57BL/6J mice, 8 weeks old, were originally obtained from Jackson Laboratory (Bar Harbor, ME) and subsequently bred in our laboratory.

Kidney slices and incubation conditions

Low glucose medium consisted of the salts of Dulbecco's MEM + 36 mg/l of glucose and 220 mg/l of sodium pyruvate. [^{3}H]Galactose was obtained from American Radiolabelled Chemicals Inc. (St. Louis, MO). The D-[1-^{3}H]galactose had a specific activity of 14 Ci/mmol.

Slices of 250-μm thickness were obtained with a McIlwain tissue slicer (Brinkman Instruments, Westbury, NY). Each incubation contained slices equivalent to one-half mouse kidney in 3 ml of low glucose medium in a 20-ml, 2 × 3 cm open plastic vial. Incubation was in a shaking waterbath at 37°C under an atmosphere of 95% O_2 and 5% CO_2.

The kidneys were removed, decapsulated, and the papillae were removed; they were then sliced and kept on ice. Medium and labeled galactose were added and the samples were incubated for the times specified. The vials were then removed, put into an ice bath, and 3 ml of ice-cold medium containing 4.5 g/l of unlabeled galactose was added. The samples were then centrifuged, the supernatant was removed, and the slices were washed with another 3 ml of cold medium and spun down again.

Isolation and HPLC of glycosphingolipids

The slices were sonicated in 2 ml of methanol per sample for 2 min and then extracted with chloroform–methanol. The glycolipid fraction was isolated and perbenzoylated with benzoyl chloride in pyridine for high performance liquid chromatography (HPLC) as previously described (4). The benzoylated glycosphingolipids were separated and quantitated by HPLC on an uncoated Zipax column with 230 nm detection and gradient elution. The gradient was 1–20% dioxane in hexane in 13 min. For determination of specific radioactivity, the eluted individual peaks were collected, taken to dryness, and further purified by reinjection onto the same gradient, collected again, and the radioactivity was determined. To completely separate peaks 6, 6a, and 7 (see Table 1 and Figs. 1 and 2), they were collected together and reinjected onto the same column with a gradient of 0.25–1.0% isopropanol in hexane in 14 min. Peaks 6, 6a, and 7 were then collected and counted. Protein was determined by the method of Lowry et al. (13).

Location of radiolabel in glycosphingolipids

The tri- and tetraglycosylceramides from a 90-min incubation were treated with 0.6 N NaOH in methanol for 1 hr at room temperature to remove the benzoyl groups. Chloroform and water were added to obtain proper conditions (14) and the upper phase was removed. The lower phase was washed two times with theoretical upper phase and then taken to dryness. The residue was dissolved in 1 ml of 1.7 N HCl in methanol and maintained at 80°C overnight. The fatty acids were extracted

with hexane and the solution was taken to dryness. The lower phase was made alkaline with ammonia and re-extracted with ether to obtain the long chain bases. After separation of the layers, all fractions were taken to dryness and radioactivity was determined.

RESULTS

Preliminary experiments

Incubation of mouse kidney slices with 10 μCi of [^{3}H]galactose for increasing time periods showed the incorporation into total GSL to be linear for 3 hr. Two-hour incubations with different quantities of [^{3}H]galactose showed incorporation to be linear from 10 μCi to 160 μCi. Acid hydrolysis of GSL and separation of fatty acids, long chain bases, and carbohydrates and subsequent determination of the radioactivity indicated that after 90 min of incubation more than 90% of the radioactivity was in the carbohydrate residues of the GSL.

Incorporation of [^{3}H]galactose into male kidney GSL

Kidney slices from male C57BL/6J mice were incubated with 100 μCi of [^{3}H]galactose for 30, 60, and 90 min. GSL was isolated by HPLC (see **Fig. 1** and **Table 1**) and the incorporation of label into the individual lipids was measured and calculated as total cpm incorporated (cpm/mg protein) and as specific activity (cpm/nmol of glycolipid) (see **Fig. 2** and **Table 2**). The monohexosylceramides with nonhydroxy fatty acid were more heavily

TABLE 1. Composition of chromatographic peaks from C57BL/6J mouse kidney[a]

HPLC Peak No.	Abbreviation for Major Components	IUPAC Nomenclature
3	Glc-Sph-Nfa[b]	GlcCer
4	Gal-Sph-Nfa	GalCer
5	Glc-Phyto-Nfa	GlcCer
6	Glc-Sph-Hfa Glc-Phyto-Hfa	GlcCer
6a	Gal-Sph-Hfa Gal-Phyto-Hfa	GalCer
7	$GaOse_2$-Sph-Nfa	$GaOse_2Cer$
8	$GaOse_2$-Sph-Hfa $GaOse_2$-Phyto-Nfa	$GaOse_2Cer$ $GaOse_2Cer$
8a	Lac-Sph-Nfa	LacCer
9	$GbOse_3$-Sph-Nfa	$GbOse_3Cer$
9a	$GbOse_3$-Phyto-Nfa	$GbOse_3Cer$
10	$GbOse_3$-Sph-Hfa	$GbOse_3Cer$
11	$GbOse_4$-Sph-Nfa	$GbOse_4Cer$
12	$GbOse_4$-Phyto-Nfa $GbOse_4$-Sph-Hfa	$GbOse_4Cer$ $GbOse_4Cer$

[a]These assignments are summaries of data previously reported (4).
[b]Sph and Phyto refer to C18-sphingosine and C18-phytosphingosine, respectively. Nfa and Hfa refer to nonhydroxy and hydroxy fatty acid, respectively.

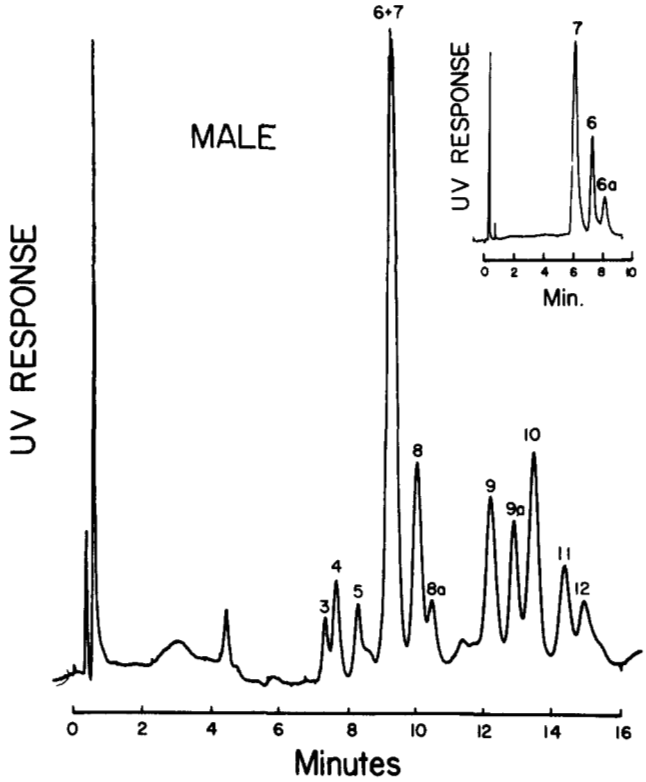

Fig. 1 HPLC of male C57BL/6J mouse kidney perbenzoylated glycosphingolipids with a gradient of dioxane in hexane as described in Methods. Peaks 6 and 7 were collected and rechromatographed with a gradient of isopropanol in hexane (see insert). See Table 1 for peak identification.

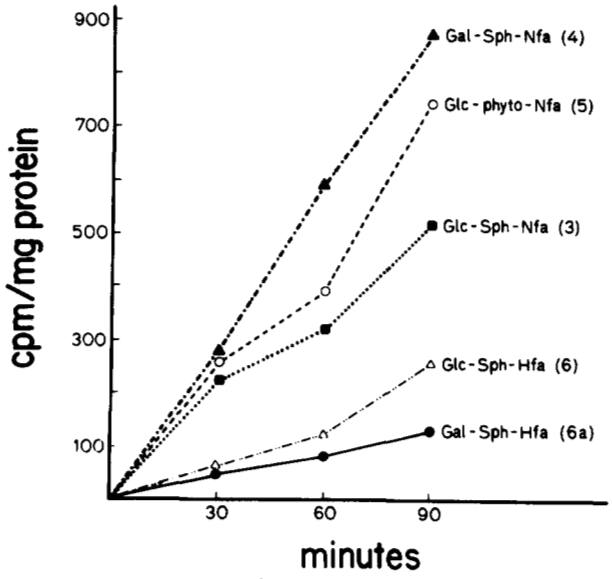

Fig. 2 Incorporation of [^{3}H]galactose into monoglycosylceramides of male kidney slices.

TABLE 2. [^{3}H]Galactose incorporation into individual glycosphingolipids of mouse kidney slices[a]

		30-min Incubation		60-min Incubation[b]		90-min Incubation	
	Peak	cpm/nmol	cpm/mg prot	cpm/nmol	cpm/mg prot	cpm/nmol	cpm/mg prot
Male	3	544(120)	224(24)	1010(35)	321(36)	1212(193)	520(37)
	4	825(16)	382(41)	1501(120)	598(43)	1735(218)	873(121)
	5	594(16)	258(43)	1179(13)	398(12)	1835(390)	749(39)
	6	78(8)	66(12)	161(0)	121(9)	274(31)	254(43)
	6a	200(53)	67(25)	374(18)	106(9)	542(40)	200(33)
	7	43(27)	53(35)	74(3)	79(4)	86(14)	119(16)
	8	92(3)	77(15)	216(11)	155(11)	285(13)	263(44)
	8a	300(94)	77(26)	600(68)	122(16)	730(140)	203(26)
Female	3	286(80)	150(27)	594(76)	314(21)	1055(173)	559(87)
	4	n.d.		n.d.		n.d.	
	5	350(103)	116(33)	971(141)	324(60)	1987(265)	661(98)
	6	44(2)	52(3)	114(8)	135(9)	204(15)	244(31)
	6a	154(13)	64(9)	357(10)	148(12)	569(42)	236(33)
	7	n.d.		n.d.		n.d.	
	8	132(16)	46(7)	317(57)	104(10)	500(56)	173(4)
	8a	193(24)	33(2)	286(39)	49(3)	506(36)	88(5)

[a]The values are averages of three determinations (± SD); cpm/nmol refers to cpm per nmol of each glycosphingolipid and cpm per mg prot refers to cpm of glycosphingolipid per mg of kidney protein; n.d., not detected.
[b]Values from only two incubations were available for male mice.

labeled than those that contained hydroxy fatty acids. This included the glucosyl- as well as the galactosylceramides. Gal-Sph-Nfa (peak 4), a lipid present only in male kidney, exhibited the highest incorporation.

Incorporation of [^{3}H]galactose into female kidney GSL

Kidney slices from female C57BL/6J mice were also incubated with 100 μCi of [^{3}H]galactose for the same time periods as for slices from male mice. The GSL was isolated as above (**Fig. 3**). The monohexosylceramides that contained nonhydroxy fatty acids were more highly labeled than those that contained hydroxy fatty acids in the female as was the case with the male. The rates of tritium incorporation into Glc-Sph-Nfa (peak 3), Glc-Phyto-Nfa (peak 5), Glc-Sph-Hfa (peak 6), and Gal-Sph-Hfa (peak 6a) were not significantly different from those in the males (Table 2 and **Fig. 4**). Gal-Sph-Nfa (peak 4) and $GaOse_2$-Sph-Nfa (peak 7), which were highly labeled in the male kidney, were not present in the female. $GaOse_2$-Sph-Hfa (peak 8) and Lac-Sph-Nfa (peak 8a) incorporated significantly more label during the same time period in the male than in the female mouse kidney slices. This relationship was not only due to increased quantities of these lipids in the tissue but was also due to increased specific activities (Table 2). The incorporation of radioactivity into glycosphingolipids after a 90-min incubation of male and female slices is presented as a bar graph in **Fig. 5.**

DISCUSSION

Incubation of kidney slices in the presence of [^{3}H]galactose led to rapid labeling of glucose and galactose in the glycosphingolipids, presumably due to the rapid equilibration of glucosyl and galactosyl nucleotide pools. The

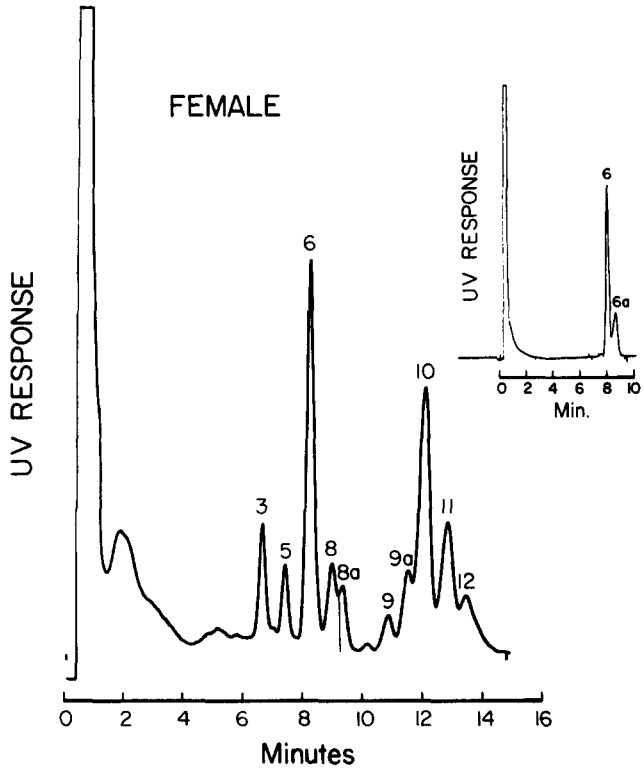

Fig. 3 HPLC of female C57BL/6J mouse kidney perbenzoylated glycosphingolipids. See Fig. 1 for conditions. The differences in retention times from those shown in Fig. 1 result from the use of a new batch of column packing material. Each new column was calibrated with a set of glycolipid standards.

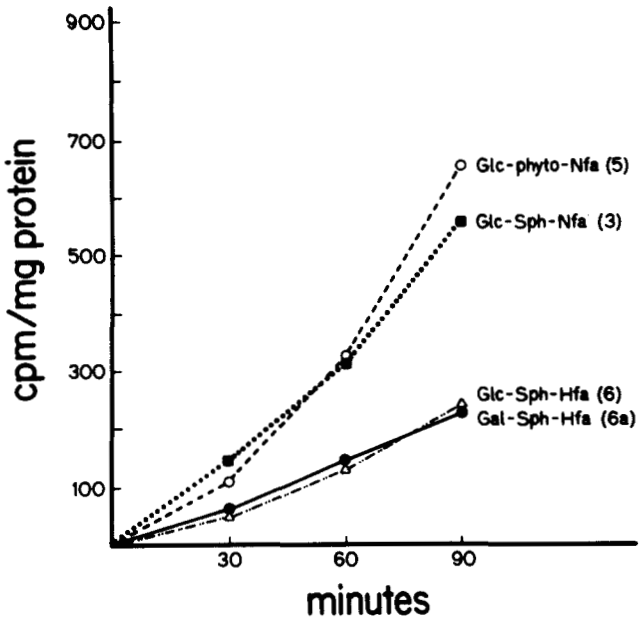

Fig. 4 Incorporation of [^{3}H]galactose into monoglycosylceramides of female kidney slices.

fact that only the carbohydrate moieties were labeled during the incubation periods used allows conclusions about relative rates of carbohydrate incorporation, assuming a common specific activity of the sugar nucleotide pools available for the synthesis of different glycosphingolipids.

The normal phase HPLC utilized in these studies provides separation of the glycosphingolipids primarily on the basis of their carbohydrate moieties and on the basis of the functional groups in the ceramides (see Table 1). This allows assessment of the differences in metabolism between these separated chromatographic species.

For the biosynthesis of the mono- and diglycosylceramides, five possible galactosyltransferase and three possible glucosyltransferase reactions are postulated. These are illustrated in **Fig. 6.**

The mono- and diglycosylceramides (Gal-Sph-Nfa, peak 4; Gal-Gal-Sph-Nfa, peak 7; and Gal-Gal-Sph-Hfa, peak 8), which were present in higher quantities in male than in female kidney, exhibited higher rates of formation in the male kidney slices (Figs. 2, 4, and 5, and Table 2). This suggests that the quantitative differences can, to a large extent, be attributed to differences in rates of synthesis. Lac-Sph-Nfa (peak 8a) also exhibited a greater rate of isotope incorporation in males, but the rates of formation of Glc-Sph-Nfa (peak 3) and Glc-Phyto-Nfa (peak 5), Glc-Sph-Hfa (peak 6) and Gal-Sph-Hfa (peak 6a) were the same in male and female kidney slices. These results suggest that the postulated biosynthetic steps Gal-T1, Gal-T2, and Gal-T3 (shown in Fig. 6) are in the proximal tubule cells, while steps Glc-T1, Glc-T2, and Glc-T3 are

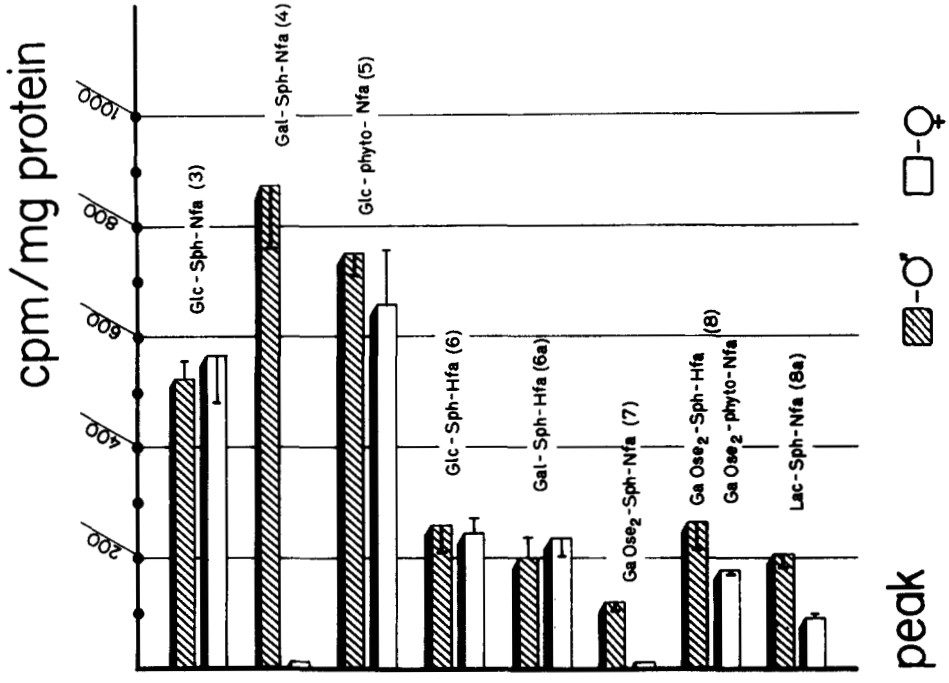

Fig. 5 Incorporatin of radioactivity from [^{3}H]galactose into glycosphingolipids of male and female kidney slices after a 90-min incubation.

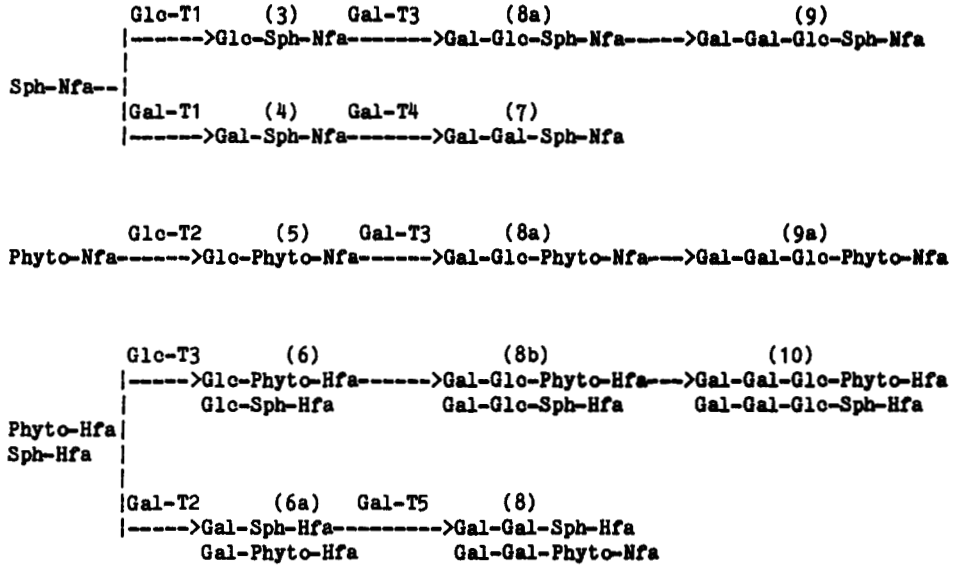

Fig. 6 Possible biosynthetic steps involved in the formation of mouse kidney neutral mono- and diglycosylceramides. Five possible galactosyltransferase (Gal-T) and three glucosyltransferase (Glc-T) steps are illustrated. The numbers in parentheses refer to chromatographic peaks (see Table 1 and Figs. 1 and 3). Because incorporation of label into the individual triglycosylceramide and LacCer-Hfa (peak 8b) was not measured, these transferase steps are not considered.

not found in these cells or are components of androgen-independent processes within the proximal tubule cells.

The apparent increased rates of formation of Gal-Sph-Nfa (Gal-T1) and Lac-Sph-Nfa (Gal-T3) without accumulation of these lipids in quantities comparable to that of the galabiglycosylceramides (peaks 7 and 8) and globotriglycosylceramides (peaks 9, 9a, and 10) suggests that the isolated Gal-Sph-Nfa and Lac-Sph-Nfa are largely intermediates rather than end products, and that the steps involved in their syntheses are rate-limiting and stimulated by testosterone. The increased rate of formation of Gal-Gal-Sph-Hfa (peak 8, Gal-T5) without a detectable difference in the rate of Gal-Sph-Hfa (peak 6a, Gal-T2) formation suggests that step Gal-T5 rather than Gal-T2 is rate-limiting and androgen-responsive.

It is of interest to note that none of the glucosyl transferase steps, Glc-T1, Glc-T2, and Glc-T3, appeared to be androgen-responsive. However, the apparent rates of these three reactions were clearly different from one another, suggesting that either three distinct enzymes or metabolic compartments or different affinities are involved. Similarly, Gal-Sph-Nfa (Gal-T1) and Gal-Sph-Hfa (Gal-T2) showed distinct rates of formation in both male and female kidney slices. The precursors involved are different ceramides and the results clearly indicate that the fatty acid and long chain base components reflect different metabolic compartments.

Previous enzymatic studies by Gray (12) and Ceccarini and Morell (11) indicated that the GSL sexual differences in the mono- and diglycosylceramide levels resulted from the increased activity of ceramide:UDP-Gal galactosyltransferase in the male kidney. They demonstrated male-female differences mainly in the activity of Hfa-ceramide: UDP-Gal galactosyltransferase in kidney homogenates and particulates. Our data indicate that the rate of synthesis of Gal-Sph-Hfa (peak 6a) is the same in male and female kidney slices, whereas the rate of synthesis of Gal-Sph-Nfa (peak 4) is high in males but is not detectable in females. The gender differences in rates of formation of the digalactosylceramides were also much greater for the nonhydroxy fatty acid-containing components ($GaOse_2$-Nfa-Sph, peak 7) than for the hydroxy fatty acid-containing $GaOse_2$-Hfa-Sph (peak 8). We postulate that the differences in the results obtained with slices, as opposed to those obtained by the earlier workers who used disrupted cell preparations, are due to compartmentalized synthesis in the intact tissue. The different rates of isotope incorporation into these components suggest that the ceramide composition of the GSL in question may be a determinant in their compartmentalization. We believe that strain differences are not relevant, as we have shown previously that several inbred strains of mice show qualitatively the same sexual differences in kidney GSL.

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