IDENTIFICATION OF 6-O-β-D-GALACTOPYRANOSYL *MYO*-INOSITOL:[1] A NEW FORM OF *MYO*-INOSITOL IN MAMMALS[2]

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SUMMARY: A disaccharide has been isolated from the mammary gland of rats on the 18th day of lactation and identified as 6-O-β-D-galactopyranosyl *myo*-inositol (6-β-galactinol). The structure was established using chemical and enzymatic methods, paper and gas chromatography, and combined gas chromatography-mass spectrometry. This is the first report of a *myo*-inositol galactoside of animal origin.

INTRODUCTION.

While separating carbohydrates of rat mammary tissue by paper chromatography, we observed that a minor component had an R_f similar to that of galactinol[1]. In this communication, we present evidence which has led us to conclude that this compound is 6-O-β-D-galactopyranosyl *myo*-inositol. To our knowledge, a disaccharide of this nature has only been detected in plants (1) and yeast (2), and this is the first report of this class in animals.

MATERIALS AND METHODS.

On the 18th day of lactation, rats were lightly ether-anesthetized, decapitated, and bled. The mammary tissue was removed and stored at -80°C. The neutral sugar fraction was obtained using the Somogyi method (3) or from a 90-100% saturated $(NH_4)_2SO_4$ fraction. In the latter case, the SO_4^{2-} was precipitated with $Ba(OH)_2$ and the supernatant treated with MB-3 resin (Rohm and Haas). In either case, the neutral sugars were separated according to the ion-exchange procedure of Wells and Dittmer (4). 6-β-galactinol was eluted in

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[1]The cyclitol nomenclature used follows: IUPAC Tentative Rules (1968), Europeam J. Biochem. 5, 1-12. Galactinol from plants is 1L-1-O-α-D-galactopyranosyl *myo*-inositol, whereas the disaccharide of animal origin is 6-O-β-D-galactopyranosyl *myo*-inositol. To avoid confusion, we propose the adoption of the trivial name, 6-β-galactinol, for the latter compound.

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the fraction between 160-210 ml. After desalting, the column fraction was streaked onto sheets of Whatman 3 MM paper, and the chromatogram (9 in.) was developed by an ascending technique using eight passes of a solvent system consisting of butanol-pyridine-water-acetic acid (6:4:3:0.3 by volume). The band with an R_f (0.41) slightly greater than galactinol (0.34) was further investigated.

Chemical hydrolysis of disaccharides was carried out in 1 N H_2SO_4 for 1 hr at 100°C. The SO_4^{2-} was precipitated with $Ba(OH)_2$, the precipitate removed by centrifugation, and an aliquot of the supernatant taken for trimethylsilation (5). Enzymatic hydrolysis was carried out by incubating the sample at 37°C for 4 hr in a mixture containing 40 mM Tris-HCl, pH 7.3, 50 mM NaCl, 50 mM KCl, and 50 µg of crystalline β-galactosidase (E.C. 3.2.1.23) (Sigma, Grade IV) in a final volume of 0.5 ml. The reaction was stopped by boiling for 5 min. After cooling, internal standard (methyl-α-D-mannosyl-pyranoside) and 3 g of MB-3 resin were added. The filtered samples were dried, trimethylsilated, and analyzed by gas chromatography (5).

Gas chromatographic separations were carried out on a Hewlett-Packard Model 402 instrument using a hydrogen flame detector. The acetates were synthesized using acetic anhydride-sodium acetate for 2 hr at 100°. Permethylation hydrolysis, and reduction of the hydrolysis products were carried out as previously published (6-8).

RESULTS AND DISCUSSION.

The similarity of paper chromatographic R_f values for galactinol and the sugar from mammary tissue indicated that their structures would be similar as did Gas-liquid chromatography of the TMS, permethyl, and peracetyl derivatives (Table I). Gas chromatography of the TMS derivative of the acid hydrolysis products showed D-galactose, *myo*-inositol, and D-glucose as products in the molar ratio of 64:50:28, respectively, and loss of the peak corresponding to the unknown. If an amount of galactose equal to *myo*-inositol is subtracted from the total galactose, a value equal to half the glucose is found and may be

indicative of contamination by one or more oligosaccharides containing glucose and galactose in a 2:1 ratio.

Additional proof that the disaccharide is composed of a hexose in glycosidic linkage with *myo*-inositol was obtained from combined gas-liquid chromatography-mass spectrometry of the permethylated and peracetylated derivatives. The mass spectra of the derivatives of galactinol and the mammary sugar are similar and can be distinguished from a di-aldohexose such as lactose on the basis of a number of fragments. In particular, the M-60 ion of the peracetylated derivative occurs at *m/e* 660 indicating a MW = 720. If the disaccharide were composed of two aldohexose units, the MW would be 678 resulting in an *m/e* of 618. No such ion appeared in the fragmentation pattern. The mass spectra will be dealt with in more detail in a forthcoming paper.

These data, in conjunction with those from acid hydrolysis, are proof that galactose and *myo*-inositol are in glycosidic linkage. It was not possible for a glucosylinositol[3] to also be present as a contaminant since the TMS derivative has a different retention time than that of the unknown sugar. Since the R_f of the mammary sugar and the retention time of the TMS derivative were not identical to those of galactinol of plant origin, three explanations were considered to account for these differences: 1) galactose is linked to a position other than the L-1 of *myo*-inositol; 2) the glycosidic linkage is β, not α, or 3) a combination of the two. The latter possibility proved to be correct as shown by the following experiments.

The position on *myo*-inositol to which galactose was linked was determined by a method similar to that used by Ballou (9) and Ueda *et al.* (10) in determining similar structures in plants. Briefly, this method involved methanolysis of the permethylated derivatives of the disaccharides and analysis of the products [pentamethyl hexose and a pentamethyl *myo*-inositol (PMM)] by gas chromatography. The position of the free hydroxyl indicates the position

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[3]A sample of authentic 6-O-α-D-glucopyranosyl-*myo*-inositol was kindly provided by Dr. Robert S. Bandurski who had obtained the sample as a gift from Dr. H. E. Carter.

Table 1. Gas chromatography of derivatives of *Myo*-inositol galactosides

Derivatives	Retention Time (Min)			
	5% SP-2401[1]		3% OV-1[2]	
	Galactinol	Mammary Sugar	Galactinol	Mammary Sugar
TMS	10.5	10.0	31.6	31.0
Permethyl	12.3	12.5	9.5	9.5
Peracetyl	--	--	21.6	21.6

[1] 5% SP-2401 on Supelcopart, 100-120 mesh, 1/8" x 6'. The temperature was 245°C and 200°C for TMS and permethyl derivatives, respectively.

[2] 3% OV-1 on Chromosorb W, 100-200 mesh, 1/8" x 6'. The temperature was 255°C, 220°C, and 200°C for TMS, permethyl, and peracetyl derivatives, respectively.

Table 2. Gas chromatography of methylated hydrolysis products of *Myo*-inositol glycosides.

Compound Methylated	Products	Retention Time (Min)
Galactinol	2,3,4,6 tetramethylgalactitol	8.1
	1,2,4,5,6 PMM	5.9
Glucosylinositol	2,3,4,6 tetramethylglucitol	9.6
	1,2,3,4,5 PMM	7.6
Unknown	2,3,4,6 tetramethylgalactitol	8.1
	1,2,3,4,5 PMM	7.6

Column was 3% OV-1, 1/8" x 6', on Chromosorb W, 100-120 mesh, and the temperature was 135°C.

to which the galactose was linked. The various positional isomers of PMM separate by gas chromatography (9, 10) and enables the position of hexose attachment to be established. In our experiments, instead of methanolysis after permethylation, we hydrolyzed the disaccharide and reduced the products which yielded a tetramethyl hexitol. This derivative separated from the PMM better than the pentamethyl pyranoside on gas chromatography.

The results of this experiment are shown in Table II. The retention time of the PMM from the mammary sugar is identical to that of the PMM from glucosylinositol (1,2,3,4,5 PMM), but different from that derived from galactinol (1,2,4,5,6 PMM). Furthermore, the mass spectra of the PMM derivatives from the mammary sugar and glucosylinositol are identical and agree with those previously published (10). Both spectra show slight differences from the PMM from galactinol which also agrees with previously published data (10). The difference is that the spectrum of 1,2,4,5,6 PMM has a mass ion at *m/e* 218 (M^{+} - CH_3OH) that is much greater in abundance than the same mass ion in the spectra of 1,2,3,4,5 PMM. In fact, as shown previously (10), all of the PMM containing the OH group in the 2,3 or 5 position have a mass ion at *m/e* 218 that is greater than 1% but the derivative with the OH in the 6 position has no ion or a very small one (< 0.2%) (10). It can also be shown from these results (Table II) that the galactose portion of the molecule was attached at the 1 position since the product obtained from hydrolysis and reduction of the permethylated derivative yielded 2,3,4,6 tetramethyl galactitol. From these results, we conclude that galactose is glycosidically linked to the 6 position of *myo*-inositol. We cannot ascertain, however, whether the linkage is 1D or 1L to *myo*-inositol.

The anomeric configuration of the galactosyl linkage was established by the hydrolysis of the mammary sugar by β-galactosidase. The products after four hours of enzymatic hydrolysis were again galactose (47 nmoles), *myo*-inositol (25.5 nmoles), and glucose (11.5 nmoles) as judged by gas-liquid chromatographic analysis. In this case, subtraction of the nmoles of *myo*-inositol from total galactose left enough galactose to represent a ratio of 2:1 with respect to the liberated glucose. Treatment of galactinol resulted in no release of products consistent with its known α-galactosidic linkage (11) and the specificity of β-galactosidase (12, 13).

The relative amount of 6-β-galactinol in the 18-day lactating rat mammary gland was approximately 5-10% that of the level of free *myo*-inositol. Gas chromatographic analysis of rat milk, human milk, and goat testes revealed a TMS

derivative with the retention time of authentic 6-β-galactinol. The tissue distribution and biosynthesis of 6-β-galactinol are under investigation.

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