Galactose Toxicity and Myoinositol Metabolism in the Developing Rat Brain*

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ABSTRACT: Interference with brain myoinositol metabolism has been identified as an effect of feeding toxic amounts of galactose to the developing rat. Pregnant rats were fed a diet containing 35% galactose approximately 10 days prior to the birth of their litters. The serum of fetuses, sacrificed 2 days before birth, contained high concentrations of galactose and galactitol. Fetal brain contained significant concentrations of galactitol and simultaneously low amounts of free myoinositol or phosphatidylinositol. Nursing young rapidly disposed of serum galactose and galactitol, but at weaning and transfer to their maternal diets, demonstrated the deposition of significant amounts of galactitol in brain tissue and a correspondingly low content of myoinositol and phosphatidylinositol. Brain slices from rats fed the galactose diet converted five to seven times less [1-^{14}C]glucose into myoinositol than comparable slices from control rats. The effect was most pronounced at 21 days of age. Significant inhibition by added galactitol of the incorporation of [2-^{14}C]myoinositol into lipid by microsomal preparations from normal rat brain was observed. Under similar conditions, the microsomal system failed to incorporate [1(6)-^{14}C]galactitol into the lipid fraction.

It has been reported from this laboratory that significant quantities of galactitol, presumably arising from the reduction of galactose through the action of aldose reductase, accumulate in the soft tissues of galactosemia patients (Wells *et al.*, 1965b; Quan-Ma *et al.*, 1966). Similarly, rats fed toxic amounts of galactose (35% of diet by weight) convert large amounts of galactose to galactitol which may be found in most tissues in excessive amounts (Quan-Ma and Wells, 1965). It has become of interest to investigate whether the galactitol thus formed acts deleteriously in the tissues concerned or represents a fortuitous route for the disposition of abnormally high levels of galactose.

We have observed that the concentration of free and lipid-bound myoinositol in tissues of both galactosemia patients (Wells *et al.*, 1965b) and rats (Quan-Ma and Wells, 1965) fed high levels of galactose were significantly lower than normal. These findings suggested that myoinositol and phosphatidylinositol biosynthesis in various tissues in the developing rat may be adversely affected by the accumulation of galactitol. We have chosen first to investigate the brain because of its highly active myoinositol metabolism (Dawson, 1954) and the recognized association of mental retardation with galactosemia (Woolf, 1962).

Experimental Section

Materials. The following materials were obtained commercially: galactitol, myoinositol, and phospholipase D from Sigma Chemical Co., St. Louis, Mo.; galactose from General Biochemicals, Inc., Chagrin Falls, Ohio; [1(6)-^{14}C]galactitol and [2-^{14}C]myoinositol from Calbiochem, Los Angeles, Calif.; [1-^{14}C]glucose from New England Nuclear Corp., Boston, Mass.; trimethylchlorosilane and SE-30 (a methylpolysiloxy gum) from General Electric Silicone Products Dept., Waterford, N. Y.; hexamethyldisilazane from Peninsular ChemResearch, Inc., Gainesville, Fla.; phosphatidylcholine, casein, Wesson salts, and vitamins from Nutritional Biochemicals Corp., Cleveland, Ohio; Dowex 1 (50–100 mesh) from J. T. Baker Chemical Co., Phillipsburg, N. J.; Amberlite MB-3 from Mallinckrodt, St. Louis, Mo.; PPO[1] and POPOP from Packard Instrument Co., Inc., Downers Grove, Ill.; and Gas-Chrom-S from Applied Science, Inc., State College, Pa.

Methods. Female rats of the Holtzman strain with positive sperm tests, 7–11 days into their pregnancies, were placed on semisynthetic diets. The control group was fed a diet of the composition: 72.8% sucrose, 18% casein, 4.0% Wesson salt mix, 5.0% cottonseed oil (supplemented with cod liver oil and α-tocopherol), 0.1% choline chloride, and 0.1% vitamin mix. The experimental group was fed a diet of the same composition except for the substitution of 35% of galactose at the expense of an equal amount of sucrose. Fetal animals were taken 1–2-days prepartum. All other

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[1] Abbreviations used: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; CTP, cytidine triphosphate.

animals were sacrificed by decapitation at specified intervals after birth for removal of tissues. Young rats were weaned at 16-days postpartum and placed on their respective maternal galactose or control diets.

Isolation of Total Lipid. Brain tissue, removed from rats at specified age intervals, was weighed and homogenized with chloroform–methanol (2:1, v/v, 20 ml/g) at room temperature (Folch *et al.*, 1957). The homogenate was filtered through a sintered-glass funnel and the residue was reextracted with additional chloroform–methanol and filtered. The combined filtrate was washed, and the solvents were evaporated from the total lipid under reduced pressure. Aliquots of the total lipid redissolved in chloroform–methanol (2:1) were later analyzed for lipid-bound inositol. The residue of brain tissue was extracted overnight with water (30 ml/g of fresh tissue).

Isolation of Free Sugars and Sugar Alcohols. The water washes from the Folch procedure and the aqueous extracts of the lipid-free residue were combined and made up to a known volume in a volumetric flask. Suitable aliquots were passed through columns of 1–2 g of mixed-bed resin (MB-3, Mallinckrodt). The sugars were washed through the column and the combined effluent was dried *in vacuo* at 30°. The residue was trimethylsilylated and carbohydrates were analyzed by gas–liquid partition chromatography (glpc) (Sweeley *et al.*, 1963; Wells *et al.*, 1964, 1965a).

Hydrolysis of Lipid-Bound and Protein–Triphosphoinositide Complex Fractions. The solvent from suitable aliquots of the total lipid fraction was evaporated and the residue was dissolved in 1 ml of 6 N HCl. Hydrolysis was accomplished by heating for 40 hr in a sealed tube at 110°. Hydrochloric acid was removed by evaporation and the residue was dried *in vacuo* over NaOH pellets for 12 hr or overnight. The residue was dissolved in small volumes of water (2–3 ml) and deionized on Amberlite MB-3 columns (1–2 g). The column was washed with three successive volumes (1 ml) of water. Water was removed from the effluent by flash evaporation in 50-ml glass-stoppered centrifuge tubes. The residue was subjected to trimethylsilylation in preparation for glpc.

The isolation of the protein–triphosphoinositide complex from the residue of extracted brain was carried out by the method of Le Baron *et al.* (1963) employing extraction with chloroform–methanol–HCl (200:100:1) for 2 hr. After acid hydrolysis of the extract, the liberated inositol was analyzed in the manner described in the previous paragraph for the hydrolysis of total lipid.

Serum and Amniotic Fluid. Serum and amniotic fluid were deproteinized by the method of Somogyi (1945). Aliquots of the filtrate were dried, trimethylsilylated, and analyzed for inositol, galactitol, and other carbohydrates by glpc (Wells *et al.*, 1964).

Gas Chromatography. The column packing was prepared from SE-30 (3%) and acid- and alkali-washed, silanized Gas-Chrom-S (80–100 mesh) by the solution-coating technique (Horning *et al.*, 1959). The F & M Corp. Model 400 gas chromatograph equipped with a hydrogen flame detector and effluent stream splitter was utilized. A 6 ft × 1/8 in. borosilicate glass U column containing the packing was maintained at 190°, and the argon inlet pressure was adjusted for optimal operating conditions.

Collection of Hexa-O-trimethylsilylmyoinositol. By means of splitting (20:1) the effluent stream, it was possible to selectively collect myoinositolhexa-*O*-trimethylsilyl ether from a mixture of sugars. At the appropriate time, labeled inositol was collected from the effluent stream by the insertion of a Pasteur pipet into the outlet port. Material which had condensed on the walls of the capillary pipet, from one or more injections, was transferred into 1-ml volumetric flasks with toluene or chloroform. Specific activity (disintegrations per minute per micromole) of the labeled inositol was determined by radioactivity measurements and mass by glpc.

Radioactivity measurements were carried out in a Packard Tri-Carb liquid scintillation spectrometer, Model 3365, with 0.6-ml toluene samples and 14.4 ml of toluene scintillation mixture of PPO and POPOP (5.0 and 0.3 g/l., respectively). Counts for each sample were corrected to disintegrations per minute by employing the external radium source in the Model 3365 spectrometer. Average efficiencies of 75% were obtained for ^{14}C.

Biosynthesis of Free and Lipid-Bound Inositol from Glucose by Brain Slices. Brains were removed rapidly from rats at specific ages, chilled in ice, and slices were prepared with a hand microtome, McIlwain and Rodnight (1962), constructed from a 1 × 4 in. microscope slide fitted with microscope cover slips to give the desired thickness. The brain slices (0.5-mm thick) were weighed and placed immediately into ice-cold Krebs–Ringer bicarbonate buffer. Slices, amounting to 0.5–0.8 g, were incubated according to Hauser and Finelli (1963) in 6.0 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing [1-^{14}C]glucose (3.0 μmoles, 5 μc) in 25-ml erlenmeyer flasks in a Dubnoff metabolic shaker at 37° for 1 hr. The flasks were gassed with 95% O_2–5% CO_2. At the end of the incubation, total lipids were extracted, washed, and free (uncombined) carbohydrates were isolated from the washes and the aqueous extracts of the lipid-free residue as described previously. Specific activity of the free and lipid-bound [^{14}C]inositol formed was determined, and the amount of [^{14}C]glucose converted to inositol was calculated.

Phosphatidylinositol Biosynthesis in Brain. The method of Thompson *et al.* (1963) was employed for the biosynthesis of phosphatidylinositol in rat brain. The tissue was dispersed in three volumes of water at 0° in a Potter–Elvehjem hand homogenizer. Nuclear and mitochondrial fractions were sedimented by successively centrifuging at 1000*g* for 10 min and 15,000*g* for 15 min. The microsomes were obtained by centrifugation of the postmitochondrial supernatant at 100,000*g* for 60 min. All centrifugations were carried out in a no. 40 rotor of the Spinco Model L preparative

TABLE I: Carbohydrate Concentration of Serum and Amniotic Fluid from Pregnant Rats and Their Progeny Fed Sucrose or Galactose.

	Diet (μmole/ml)			
	Galactose			Sucrose[b]
Sample[a]	Glucose	Galactose	Galactitol	Glucose
Maternal serum	4.12	4.00	0.44	5.40
Amniotic fluid[c]	0.97	3.20	1.40	0.97
Fetal serum	2.65	3.96	1.22	7.08
Progeny serum (days postpartum)				
9	2.71	0	0	3.79
19	3.16	2.90	<0.32	3.80
21	2.34	3.64	<0.32	3.80
30	2.76	2.56	0.69	2.86
32	2.62	2.02	0.32	2.67
54	2.10	3.42	<0.32	3.56
75	5.08	7.50	<0.32	6.38

[a] All values reported are the average of duplicate determinations of pooled serum from four to six rats. [b] Neither galactose nor galactitol was present in the serum or amniotic fluid. [c] Duplicate analysis of pooled amniotic fluids from two rats.

ultracentrifuge. Microsomal pellets were suspended in 0.25 M sucrose (1–1.2 g of brain/3.0 ml) before incubation. Phosphatidic acid was obtained by the action of phospholipase D on crude animal lecithin according to the procedure of Einset and Clark (1958).

In all experiments, aliquots (0.02 ml) of the microsomal suspension derived from 12 mg wet weight of brain were pipetted into 12-ml glass-stoppered centrifuge tubes containing 0.1 M Tris buffer (pH 7.4) (50 mM), potassium phosphate (pH 7.4) (10 mM), CTP (1.0 mM), $MnCl_2$ (16 mM), [2-^{14}C]inositol (125 mμmoles), and 125 mμmoles of phosphatidic acid dispersed in Tween 80 (0.025 mg/ml) for a final volume of 0.3 ml. Incubation was at 37° for 1 hr. At the end of the incubation period, the vessels were chilled in an ice bath, and 5 ml of chloroform–methanol (2:1, v/v) was added.

The chloroform–methanol extracts were purified by the method of Folch *et al.* (1957). Myoinositol (95 mg/100 ml) was added to the "upper-phase" solvent (chloroform–methanol–water, 3:48:47, v/v). After six washes with this modified "upper-phase" solvent, radioactivity remaining in unincubated samples was found to be negligible. For studies of the effect of galactitol added to the incubation medium, microsomes were isolated as described by Thompson *et al.* (1963) and incubated in a final volume of 0.3 ml containing the following components: CTP (1.0 mM), $MnCl_2$ (16 mM), phosphatidic acid (0.125 μmole), inositol (0.125 μmole), Tris (pH 7.4) (50 mM), KH_2PO_4 buffer (pH 7.4) (10 mM), and galactitol (0.032–0.25 μmole). Lipid radioactivity was determined as described previously.

Incorporation of [1(6)-^{14}C]Galactitol into Lipid. The incubation medium, lipid isolation, and radioactivity measurements employed were essentially the same as those described in the previous section for [^{14}C]myoinositol incorporation. The only exception was that 0.125 μmole (4.0 μc) of [1(6)-^{14}C]galactitol was substituted for an equal amount of labeled inositol.

Results

Amniotic Fluid and Serum. The uncombined carbohydrate content of serum and amniotic fluid from rats fed diets containing galactose and sucrose are listed in Table I. Glucose was the only carbohydrate detected in the amniotic fluid removed from rats fed the diet containing sucrose, whereas glucose, galactose, and galactitol were isolated from that of rats fed galactose. The galactose concentration of maternal and fetal serum was equivalent, but the galactitol content of the fetal serum was three times that of the mother. The glucose concentration of fetal serum from control rats was somewhat higher than that from maternal glucose (7.08 *vs.* 5.4 μmoles/ml). The glucose concentration in serum from both maternal and fetal animals fed galactose were lower than that of controls; the lowest occurred in the serum of fetuses from mothers fed galactose (2.65 μmoles/ml). Suckling rats were capable of rapidly clearing their serum of detectable amounts of galactose and galactitol; nevertheless, their glucose concentrations generally remained below that of the controls. Animals were weaned 16 days after birth and placed on the respective maternal diets. Despite high concentrations of galactose in the serum of young rats fed galactose, the galactitol content never reached that found in the fetal serum

TABLE II: Concentration[a] of Free Galactitol and Myoinositol in Brains of Rats Fed Diets Containing Galactose or Sucrose.

Age of Rats (days)	Diet (μmole/g)		
	Galactose		Sucrose
	Galactitol	Myoinositol	Myoinositol[e]
−2[b]	5.26	4.20	5.73
1[c]	0.60	2.10	2.25
9	0.10	2.10	3.10
15	0.12	2.19	3.68
17[d]	2.00	2.27	3.08
19	4.35	2.10	2.60
21	4.10	4.20	4.80
32	6.50	4.00	4.40
38	4.92	4.75	5.53
54	5.42	2.80	5.50
75	5.92	2.70	4.60

[a] The values reported were the averages of duplicate analyses of Folch washes from the lipid extract of pooled tissue samples of four to six brains. [b] Fetal animals, 2-days prepartum. (Pregnant females received the diets 15–16 days prior to birth of the litter.) [c] Females continued on diets; progeny are suckling. [d] Progeny, weaned at 16 days of age, were fed corresponding maternal diets. [e] No other free sugar was detectable.

TABLE III: Effect of Feeding a Diet Containing 35% of Galactose on Lipid-Bound Inositol in the Brain of the Rat.

Days after Feeding	Diet (μmole/g of fresh tissue)[b]	
	Galactose	Sucrose
−2[a]	0.66	0.97
1[c]	0.54	0.62
7	0.57	0.64
15	0.49	0.67
17[d]	0.80	1.54
19	0.91	1.27
21	1.06	1.60
32	1.13	1.62
54	1.27	1.62
75	1.23	1.65

[a] Fetal animal 2-days prepartum. (Pregnant females received the diets 15–16 days prior to birth of the litters.) [b] The values reported were the averages of duplicate analyses of lipid extracts of a pooled tissue sample of four to six brains. [c] Females continued on diets; progeny are suckling. [d] One day after weaning.

from corresponding animals.

Myoinositol and Galactitol Content of Brain. Myoinositol was the sole free polyol detected in significant quantity in the brains from rats fed the control diet (Table II). Following a sharp decrease in myoinositol concentration observed in brains from 1-day-old rats relative to that of fetal brain, increasing concentrations were found in brains from both dietary groups of rats up to 38 days old. Myoinositol quantities dropped after 38 days, the more remarkable drop occurring in the brains of rats fed galactose. The galactitol concentration of rats ingesting galactose was higher in the fetal brain than in those from rats 1–15 days old. At 16 days of age, when the young began to eat toxic amounts of galactose, galactitol again appeared in the brain and remained at a high concentration as long as galactose feeding was maintained.

Lipid and Protein-Bound Myoinositol. The lipid phosphatide myoinositol content of brains from rats fed the two experimental diets is compared in Table III. The greatest difference in lipid-bound myoinositol concentration of the two groups occurred at 17-days postpartum. Analysis of the myoinositol content of the protein–triphosphoinositide fraction of the developing brains from all rats by glpc revealed very low levels of myoinositol; however, brains from rats fed the

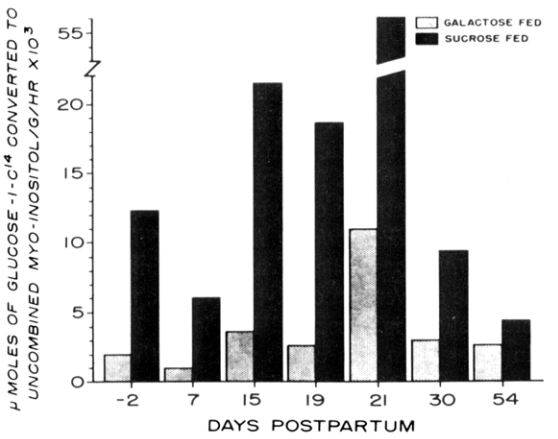

FIGURE 1: Effect of galactose feeding on myoinositol biosynthesis from [1-^{14}C]glucose. Brain slices (0.5 g) were incubated in Ringer bicarbonate buffer containing 4.0 μc of [1-^{14}C]glucose. After incubation for 2 hr at 37° in an atmosphere of 95% O_2 and 5% CO_2, lipids were extracted by addition of chloroform–methanol (2:1). The total free myoinositol content of the combined Folch washes and aqueous extracts of the lipid-free residue was determined by gas–liquid partition chromatography. The volatile trimethylsilyl derivative of myoinositol was collected from the effluent stream of the gas chromatograph and its specific activity was determined. Conversion of added [1-^{14}C]glucose to myoinositol was calculated from the specific activity and total myoinositol present.

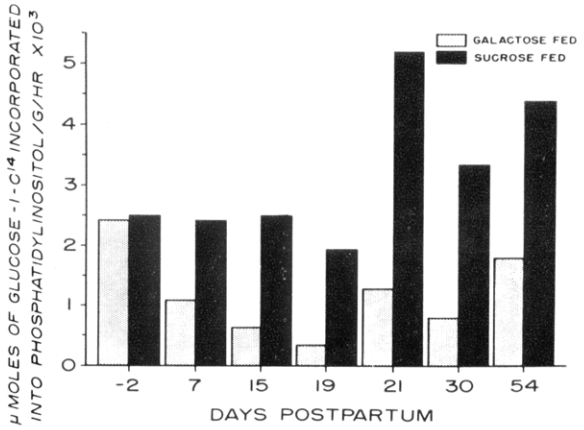

FIGURE 2: Effect of galactose feeding on conversion of [1-^{14}C]glucose into phosphatidylinositol in brain slices. Aliquots of the lipid extract obtained as described in Figure 1 were hydrolyzed with 6 N HCl and the total lipid-bound myoinositol was determined by gas–liquid partition chromatography. The trimethylsilyl derivative was collected from the effluent stream of the gas chromatograph and specific activity was determined. Conversion of glucose to phosphatidylinositol was calculated from the specific activity and total lipid-bound myoinositol which was present.

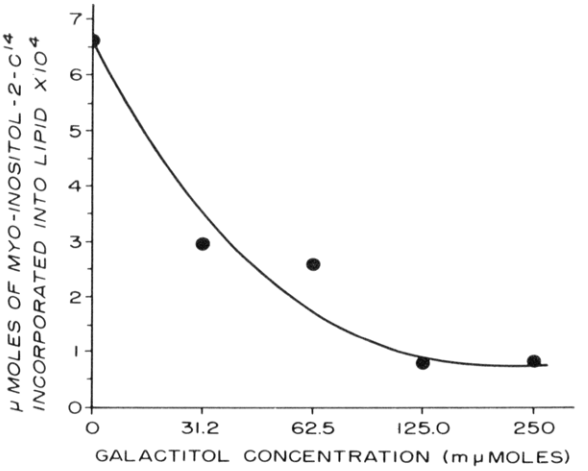

FIGURE 3: Effect of the presence of galactitol on the incorporation of [2-^{14}C]inositol into lipid in a cell-free preparation. Incubation medium included microsomes isolated as described by Thompson *et al.* (1963) and incubated with CTP (1.0 mM), $MnCl_2$ (16 mM), phosphatidic acid (0.125 μmole), inositol (0.125 μmole), 50 mM Tris (pH 7.4)–10 mM KH_2PO_4 buffer (pH 7.4), and galactitol (0.032–0.25 μmole). After incubation, lipid was extracted with chloroform–methanol (2:1) and washed until the wash water was free of radioactivity. Lipid radioactivity was determined as described in the Experimental Section.

sucrose diets contained approximately twice that of brains from rats fed galactose in every comparable group tested. Only traces of myoinositol from the protein–triphosphoinositide complex were detected in fetal brains from rats fed either diet. The viscous protein fraction of several brains, isolated by extraction of the lipid-free residue with 0.03 N HCl, was analyzed after acidic hydrolysis for myoinositol content by glpc. In all cases, only traces of myoinositol were detected.

Myoinositol Biosynthesis in Brain Slices. Effect of Dietary Galactose. The effect of feeding galactose to rats on the conversion of [1-^{14}C]glucose to myoinositol in brain slices is presented in Figure 1. At all ages, brain slices from rats which were fed the control diet, converted five to seven times as much glucose to myoinositol as did those from rats which were fed galactose. The relative effect of feeding galactose decreased after 21-days postpartum and at subsequent periods of growth when general myoinositol synthesis declined.

Results of the incorporation of [1-^{14}C]glucose into phosphatide-bound myoinositol by brain slices are plotted in Figure 2. Fetal brain slices of both groups converted equal amounts of [1-^{14}C]glucose to monophosphoinositide. After birth, however, greater amounts of glucose were converted to monophosphoinositide by brain slices from rats fed sucrose than from those fed galactose.

Inhibition of Phosphatidylinositol Biosynthesis by Galactitol. Incorporation of [2-^{14}C]myoinositol into lipid was studied by incubating a microsomal preparation from brains of rats fed stock diets with the required cofactors according to Thompson *et al.* (1963) (Figure 3). Significant inhibition of the incorporation of labeled myoinositol into lipid by added galactitol occurred. For example, in the presence of 32.0 mμmoles of galactitol, the incorporation of 125 mμmoles of labeled myoinositol was inhibited by 50%. As inhibitor concentration increased, there was a proportional decrease in the incorporation of myoinositol into phosphatides. Utilizing a Lineweaver–Burk plot (Figure 4), preliminary results indicated that galactitol

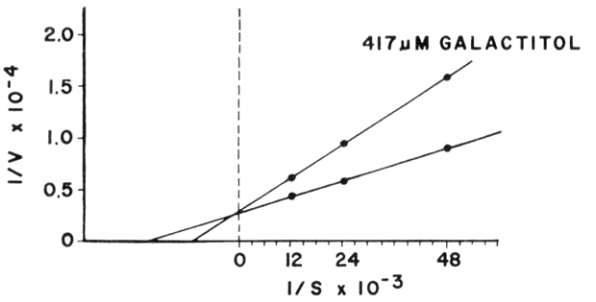

FIGURE 4: Lineweaver–Burk plot of reciprocal velocity as a function of reciprocal substrate concentration. Incubation medium and incorporation of [2-^{14}C]inositol into lipid was determined as in Figure 3.

is a competitive inhibitor of phosphatidylinositol biosynthesis. These values are based on the assumption that the rate-limiting step is related to the initial velocities of this complex system.

Failure to Incorporate [1(6)-^{14}C]Galactitol into Lipid. Previously, Paulus and Kennedy (1960) were unable to obtain incorporation of galactitol into lipid with microsomal preparations of liver and kidney. Because of the inhibitory action of galactitol on the incorporation of [2-^{14}C]myoinositol into phosphatidylinositol, it was considered possible that an abnormal lipid incorporating galactitol; *e.g.*, phosphatidylgalactitol might be synthesized by preparations from rat brain. [1(6)-^{14}C]Galactitol was not found to be incorporated into the lipid isolated from incubation of a microsomal preparation from rat brain in phosphate buffer (pH 7.4) containing $MnCl_2$, phosphatidic acid, CTP, and [1(6)-^{14}C]galactitol.

Discussion

The data reported here support the hypothesis that the fetus has the capability to synthesize galactitol. Thus, fetal serum was shown to have greater amounts of galactitol than maternal serum. The high concentration of galactitol in amniotic fluid was consistent with the recognized slow transport of the acylic polyols across a cellular membrane (Wick and Drury, 1951). In contrast to fetal excretion, maternal renal clearance, characterized by poor reabsorption of galactitol by kidney tubules, would lead to a rapid disappearance of galactitol from maternal serum.

Litters from female rats fed diets containing 35% of galactose often appeared abnormal, many not surviving beyond 24 hr. The physiological period of nursing of those young surviving the toxic pregnancy period was associated with a return to approximately normal concentrations of free and lipid-bound myoinositol concentrations in brain. At 16-days postpartum, a period coinciding in rats with the onset of myelination and increasing rate of incorporation of labeled myoinositol into phosphatidylinositol, galactose feeding profoundly inhibited both myoinositol and phosphatidylinositol biosynthesis.

The mechanism of inhibition of myoinositol biosynthesis is unclear since the inhibition may be caused by galactose itself, galactose 1-phosphate, galactitol, or some other metabolite of galactose. Galactose 1-phosphate has been reported to inhibit phosphoglucomutase (Najjar and Pullman, 1954) which would have an effect on the glucose 6-phosphate concentrations derived from glycogen *via* glucose 1-phosphate. In the brain-slice experiments reported here, however, the labeled precursor of myoinositol was glucose. Thus, any of the enzymatic steps leading from glucose to myoinositol, those involving the formation of glucose 6-phosphate, D-myoinositol-1-phosphate (Eisenberg and Bolden, 1965), etc., are implicated.

It was reasoned that galactitol might be an inhibitor of myoinositol incorporation into monophosphoinositide because the lowest concentration of monophosphoinositide relative to that of the control occurred in rats fed galactose at a time when galactitol accumulation was appreciable. This hypothesis was supported by the demonstration of inhibition of myoinositol incorporation into lipid by a particulate system of rat brain although isotopic galactitol could not be found in the lipid fraction derived from the cell-free system. The mechanism of the inhibition of myoinositol synthesis, a defect not previously detected, may be more directly related to the accumulation of galactose derivatives in the brains of rats than to the direct toxic action of galactitol. However, galactitol was shown to inhibit incorporation of myoinositol into phosphatidylinositol, identifying a specific site in brain metabolism which is susceptible to the accumulation of galactitol. These results suggest that galactose toxicity is related to more than one mechanism. The total extent to which galactitol plays a role in the malfunction of the brain will require further study.

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