myo-INOSITOL METABOLISM DURING DEVELOPMENT AND LACTATION IN THE RAT

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ABSTRACT

myo-INOSITOL METABOLISM DURING DEVELOPMENT AND LACTATION IN THE RAT

By

Louis Eugene Burton

The disposition of myo-inositol in rat tissues was examined during the last four days of gestation, during neonatal development and during lactation. Developmental profiles were obtained for the enzymes D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) and L-myo-inositol-1-phosphate: L-myo-inositol phosphatase (EC 3.1.3.25) in fetal and neonatal liver and brain, maternal liver and mammary tissue, and placenta. Developmental free myo-inositol levels were measured for plasma, liver and brain of the fetal and neonatal rat and for plasma, liver, placenta and mammary tissue of the maternal rat.

Enzymatic studies, notably in fetal liver, suggested that elevated fetal plasma levels (7-8-fold that of the dam) of myo-inositol may originate from liver enzymatic synthesis and subsequently be replaced by nutritional (milk myo-inositol and mammary myo-inositol synthesis) sources during neonatal development. Fetal plasma myo-inositol levels were not significantly affected by dietary myo-inositol deprivation, however, 2-deoxy-D-glucose administration was shown to depress myo-inositol levels in fetal plasma and most fetal tissues (in the presence or absence of dietary myo-inositol) suggesting that biosynthesis accounts for at least 60% of the high fetal content of myo-inositol.

Comparisons of fetal, weanling and adult rat tissue/plasma myo-inositol ratios suggested developmental increases in the ability of tissues to retain intracellular myo-inositol.

Dietary studies were undertaken to examine the effects of dietary myo-inositol deprivation on the growth and development of fetal, neonatal and young adult rats and on lactation in dams. These studies suggested that intestinal flora contribute significant amounts of myo-inositol to the animal during deprivation.

Dietary myo-inositol strongly influenced the levels of myo-inositol in the milk, mammary tissue and liver of the lactating dam. Levels of milk 6-β-galactinol were also shown to be directly related to levels of milk myo-inositol in rats and in a single human subject. The feeding of a myo-inositol deficient diet and the physiological stress of lactation produced a fatty liver after four days of lactation which was alleviated by myo-inositol supplementation or by cessation of lactation. Ninety-seven percent of the lipid deposited in the myo-inositol deficient lactation-induced fatty liver was shown to be triglyceride. Plasma of lactating rats fed a myo-inositol deficient diet had reduced levels of lipoprotein-associated lipids and elevated free fatty acid levels during the course of lactation and, subsequently, elevated lipoprotein-associated lipids levels and normal free fatty acid levels during involution. These observations have suggested that myo-inositol deficient lactation-induced fatty liver results from a block in holo-lipoprotein synthesis or secretion caused by a reduction in liver phospholipids and phosphatidylinositol during myo-inositol deprivation.

Additional work was carried out examining the effects of 2-deoxy-D-glucose and 5-thio-D-glucose administration on myo-inositol levels

in testes and liver of mice. The 6-phosphate of 5-thio-D-glucose was enzymatically synthesized and shown to be a competitive inhibitor (K_i=0.33 mM) of D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4, D-glucose-6-phosphate, K_m=0.51 mM) in vitro. In vivo administration of 2-deoxy-D-glucose resulted in reduction of myo-inositol levels in the testes and liver of mice while 5-thio-D-glucose administration resulted in significant elevations in testicular myo-inositol. Preliminary evidence suggests that in 5-thio-D-glucose treated animals, myo-inositol elevations are a result of the indirect effects of 5-thio-D-glucose administration on glucose metabolism and that inhibition of myo-inositol biosynthesis by 5-thio-D-glucose-6-phosphate in vivo does not occur.

myo-INOSITOL METABOLISM DURING DEVELOPMENT AND LACTATION IN THE RAT

By

Louis Eugene Burton

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DEDICATION

I wish to personally dedicate this work to Iris Ann, my wife. May our lives benefit from this accomplishment as much as we hoped. It will always be as much hers as it is mine.

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
C, CE	Cholesterol, cholesterol esters
CNP	2', 3'-Cyclic nucleotide 3'-phosphohydrolase
2DG, 2DG-6-P	2-Deoxy-D-Glucose and its 6-phosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
FFA	Free fatty acids
G-6-P	D-Glucose-6-phosphate
IU	International unit
K_i	Inhibition constant
K_m	Michaelis constant
NAD^+, NADH	Oxidized and reduced nicotinamide adenine dinucleotide
$NADP^+$, NADPH	Oxidized and reduced nicotinamide adenine dinucleotide phosphate
PI	Phosphatidylinositol
PL, PLP	Phospholipid, phospholipid phosphorus
S.D.	Standard deviation
TCA	Trichloroacetic acid
TG	Triglyceride
5TG, 5TG-6-P	5-Thio-D-glucose and its 6-phosphate
Tris	Trishydroxymethylaminomethane
V_{max}	Maximum velocity

INTRODUCTION

A brief survey of the literature concerning myo-inositol is presented in order to supplement a discussion of the objectives and approach of this project.

LITERATURE SURVEY

Although the existence of cyclitols and especially myo-inositol have been known for more than 100 years, the study of their biochemistry has developed only in the last 40 years. Interest in myo-inositol increased following the discoveries implicating its importance to living systems; i.e., growth factor or vitamin qualities, and its role as a component of phospholipids.

Several reviews of interest have since been written including those by Weidlein (1) on the biochemistry of cyclitols and a more recent survey by Posternak (2). Another publication (3) worthy of attention is a survey of recent advancements in the area of cyclitols and phosphoinositides assembled by the New York Academy of Sciences.

myo-Inositol and its Nutrient Role. After the discovery of myo-inositol by Scherer (4) in 1850, most of the initial work was directed at the isolation of myo-inositol from a large variety of tissues (5). The more recent studies of this compound have centered around its essential nutrient role in microorganisms, mammals, and man. Although it has been proven to be an essential nutrient of yeast and fungi (6-9), its vitamin-like function in mammals has not yet been established. Eagle et al.(10, 11) have demonstrated that myo-inositol is essential to the growth and survival of 18 normal and cancerous human cell lines and two normal mouse cell lines. More recently (12), they have also presented evidence that of 22 mammalian cell strains, only one can be

grown indefinitely on myo-inositol free mediums.

The role of myo-inositol as a vitamin in animals was first examined by Woolley (13) in 1940. In this study, he demonstrated that mice on a myo-inositol deficient diet displayed symptoms of inadequate growth and alopecia, and death followed in two to three weeks. In later studies (14), he found that supplementation of the diet with 100 mg of myo-inositol or phytic acid/100 g of diet relieved the deficiency symptoms. Martin (15), however, found less chronic alopecia in mice fed the deficient diet and found the deficiency symptoms somewhat remedied by pantothenic acid. Woolley (16) confirmed this observation in later experiments and explained the apparent discrepancy by synergism between pantothenic acid and myo-inositol (17). As a result of this work, requirements for myo-inositol by other species have been investigated.

Studies on the requirements of the rat for myo-inositol gave contradictory results (19-26). In earlier studies, diets were found to contain contaminating myo-inositol in starch and casein (27, 28) and sucrose (26). myo-Inositol supplied by intestinal flora (29) has also been shown to be an important exogeneous source in dietary studies. These sources undoubtedly contributed to the earlier contradictions and the difficulty of interpretation of studies in this area. In addition, endogenous contributions, which include in vivo synthesis (28) and possible tissue mobilization of myo-inositol, present problems in the study of its nutrient or vitamin-like role in growth and specific tissue requirements.

Biosynthesis of myo-Inositol. Suspicion that animals may have the capability to synthesize myo-inositol in vivo began with Needham's observations of continued inositoluria in rats maintained on low myo-inositol diets (30). myo-Inositol biosynthesis in vivo, however, remained uncertain due to inconsistent results (26) and suggestions that

intestinal flora may have been a potential source (17). Later work in rats and mice utilizing radioactive glucose as a precursor and intestinal antibacterial agents (28, 31) or germ-free animals (32) definitively demonstrated the biosynthesis of myo-inositol from glucose in mammalian tissues.

Although biosynthesis in animals had been shown, neither the sites nor the pathway of myo-inositol synthesis had been elucidated. In experiments utilizing tissue slices and radioactive glucose, Hauser and Finelli (33) demonstrated the incorporation of ^{14}C from glucose into myo-inositol in liver, kidney and brain of the rat. At the same time, an in vitro enzymatic biosynthesizing system was being uncovered by Eisenberg and Bolden in rat testis (34 - 36), Chen and Charalampous in yeast (37, 38) and Pina and Tatum in Neurospora crassa (39).

Discovery of biosynthetic activity for myo-inositol resulted in attempts to elucidate the mechanism of the enzymatic formation of myo-inositol from glucose. The biosynthesis of myo-inositol from D-glucose-6-phosphate is catalyzed by D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (synthase; EC 5.5.1.4) and L-myo-inositol-1-phosphate phosphatase (phosphatase; EC 3.1.3.25) (40). The synthase has been shown to be rate limiting with a requirement for NAD^+ (41) and has been the most extensively studied of the two enzymes regarding properties (42 - 44) and mechanism (40, 45 - 47). The synthase from higher forms of life has been classified as a type I cycloaldolase requiring an intramolecular oxidation-reduction sequence at C-5 using NAD^+ and a Schiff's base intermediate (41, 44, 45). Synthase from N. crassa and yeast is a type II cycloaldolase requiring Zn^{2+} (42, 48).

myo-Inositol and Lipids. The major role myo-inositol is known

to play is as a component of tissue phospholipids. The majority of studies involving phosphoinositides have been centered in brain tissue. The three classes of known phosphoinositides include monophosphoinositide, diphosphoinositide and triphosphoinositide. Excellent reviews concerning biosynthesis, chemistry and metabolism of this group of phospholipids have recently been presented (49, 50). Suggested physiological roles of polyphosphoinositides in neural membranes have included affinity for divalent cations (51) and possible involvement in postsynaptic events during transmission in sympathetic ganglia (52).

Since 1944, conflicting reports have been published on the role of myo-inositol as a lipotropic agent. Gavin and McHenry (53) first reported the lipotropic action of myo-inositol prevented formation of a special biotin fatty liver. Other workers supported these observations and reported interactions and comparisons between myo-inositol and choline (20, 54, 55) in alleviating fatty livers as well as showing myo-inositol's ineffectiveness against thiamine type fatty livers (53). Best and his co-workers (56), however, differed with these views and could produce no lipotropic action that could be attributed to myo-inositol. Hegsted, et al. (57, 58) produced a dietary oil dependent intestinal lipodystrophy in female gerbils by withholding myo-inositol. More recently, Hayashi, et al. (59) demonstrated fatty liver formation in male weanling rats in the presence of sulfa drug when fed a myo-inositol deficient diet containing a saturated cottonseed oil. Utilizing a diet similar to Hayashi, Burton and Wells (60) have produced a fatty liver in female rats which is dependent upon myo-inositol deficient diet and lactation and in the presence of an unsaturated fat source. Work to elucidate the characteristics of the myo-inositol deficient fatty liver

by Burton and Wells (this thesis) and others (59a, 61) suggests it to be very similar to the fatty liver produced by choline deficiency.

myo-Inositol Metabolism and Other Proposed Functions. This brief survey would not be complete without a discussion of myo-inositol catabolism and suggested functions beyond those previously mentioned. The catabolism of myo-inositol is believed to progress through the pentose cycle via the glucuronic acid pathway (62, 63). These reactions ultimately lead to D-xylulose-5-phosphate, glycolytic intermediates and potential gluconeogenesis (62).

Proposed functions of myo-inositol secondary to its major role as a component of membrane phosphoinositides are many and varied. Some of these functions include: cofactor of galactosyl transferase in the synthesis of verbascose (64) and stachyose (65); a contraction factor in mitochondria as a phosphoinositide (66); a role in cellular polarity (67-70); as a substrate in the production of 6-β-galactinol in milk and mammary gland (71), uronic and pentose units and phytic acid in plants (72), indoleacetic acid esters in plants (73) and the synthesis of bluenomycin (74) and streptomycin (75); and a potential role as a stabilizer of microtubules (76). Finally, major observations have been made regarding myo-inositol, diabetes and insulin release. Diabetes has been shown to be accompanied by marked increases in plasma and urinary myo-inositol levels (77) and these elevations as well as the resulting decrease in nerve tissue myo-inositol levels may be responsible for the impaired motor nerve conduction velocity observed in the peripheral nerves of diabetics (78). With regard to insulin release, myo-inositol has been proposed to be involved in the process as the phosphoinosidide (79) and some preliminary work in that area has begun (80).

OBJECTIVES AND RATIONALE

Aside from the apparent role which myo-inositol phosphatides play as important membrane constituents (49-52) other potential functions which have been examined include: a lipotropic function (53-59), a growth factor for microorganisms (6-9) and mammalian cell lines (10-12), and an animal vitamin (81).

It was the goal of this research to examine the role of exogenous and endogenous sources of myo-inositol and their effect on growth, development and myo-inositol metabolism in the rat. Initial work (Chapter I, 82) in these studies involved an investigation of the origin of the concentration difference between fetal and neonatal plasma and maternal plasma. As a first step in the understanding of the relatively higher levels of myo-inositol in fetal and neonatal rat plasma as compared to the levels in maternal plasma, the examination of the developmental changes in the level of this compound was essential. A determination of the effects of the two major sources, biosynthesis and diet, would aid in evaluating the significance of these differences. The approach used here involved examining changes in the levels of milk myo-inositol during lactation and myo-inositol and its biosynthetic enzymes in plasma and tissues during the course of fetal and neonatal development. In addition, dietary supplementation versus denial of myo-inositol was used as an approach to ascertain what proportion of myo-inositol in the adult and developing rat might be of dietary origin and what proportion might be due to de novo synthesis.

During the course of the dietary studies, a myo-inositol deficient lactation-induced fatty liver was discovered that was prevented by dietary supplements of myo-inositol or by termination of lactation (Chapters III, IV). Recent work, therefore, has emphasized the

character of the lipid deposits as an approach to understanding the mechanism of formation of the fatty liver. Investigation of this problem may have relevance to humans and especially maternal nutrition during lactation. Studies regarding the relationship between dietary and milk levels of myo-inositol may prove to be of interest. Infants who cannot be nursed normally (for example, premature and galactosemic infants) may require more than the low levels of myo-inositol observed in bovine milk and commercial formulas (82).

The latter portion of this thesis set out to examine potential inhibitors of biosynthesis of myo-inositol in order to supplement the dietary work and aid in the interpretation of the role of myo-inositol in development. Establishment of 5-thio-D-glucose-6-phosphate as a new in vitro inhibitor of the biosynthesis of L-myo-inositol-1-phosphate and the study of the effects of 5-thio-D-glucose and 2-deoxy-D-glucose (the 6-phosphate is a known potent inhibitor of myo-inositol synthesis in vitro (83)) administration on myo-inositol in the mouse culminate the studies presented here. With the establishment of in vivo inhibitors of myo-inositol biosynthesis, a better understanding of the role biosynthesis plays in myo-inositol metabolism should develop.

ORGANIZATION

The text of this thesis has been presented as chapters, each following a format used in most biochemical journals. Several of the chapters or portions thereof represent material already published or submitted for publication at the present time and reflect the literary style of the journal for which they were written. Chapter I, with the exception of the 2-deoxy-D-glucose work and the tissue myo-inositol survey, is presented as it appears in Developmental Biology 37, 35-42

(1974), under the title of "Studies on the Developmental Pattern of the Enzymes Converting Glucose-6-Phosphate to myo-Inositol in the Rat" by Louis E. Burton and William W. Wells. Chapter III and Chapter V have been submitted to the Journal of Nutrition for publication. Chapter III has been submitted under the title of "myo-Inositol Metabolism During Lactation and Development in the Rat. The Prevention of Lactation-Induced Fatty Liver by Dietary myo-Inositol" by Louis E. Burton and William W. Wells. Chapter V represents a selected portion of a submitted article entitled "myo-Inositol Metabolism in the Neonatal and Developing Rat Fed a myo-Inositol Deficient Diet" by Louis E. Burton, Rita E. Ray, James R. Bradford, Joanne P. Orr, Jeffery A. Nickerson and William W. Wells. Chapter VI is presently being submitted to Archives of Biochemistry and Biophysics for publication under the title of "Studies on the Effect of 5-Thio-D-Glucose and 2-Deoxy-D-Glucose on myo-Inositol Metabolism in Mice and Inhibition by 5-Thio-D-Glucose-6-Phosphate of L-myo-Inositol-1-Phosphate Synthesis, in vitro" by Louis E. Burton and William W. Wells.

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CHAPTER I

STUDIES ON THE DEVELOPMENTAL PATTERN OF THE ENZYMES CONVERTING GLUCOSE-6-PHOSPHATE TO myo-INOSITOL IN THE RAT

ABSTRACT

The myo-inositol level of plasma was determined during pre- and post-natal development of the rat. Fetal concentrations exceeded those of maternal rats by nearly 10-fold. Immediately after birth, the myo-inositol level decreased but was maintained at values 3-4 times that of the lactating dams. The cyclitol content of rat milk was high and rose during lactation to a maximum of 1.6 mM.

The biosynthesis of myo-inositol from glucose-6-phosphate is catalyzed by D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) and L-myo-inositol-1-phosphate phosphatase (EC 3.1.3.24). The activity of both enzymes was monitored in fetal and neonatal liver, maternal liver, placenta, and mammary gland. Results indicated that fetal liver accounted for over 48% of the total fetal carcass synthase and 26% of the total fetal carcass phosphatase activity. Developmental changes correlated well with the pattern of myo-inositol in fetal rat plasma. Similarly, the enzymes of the myo-inositol biosynthetic pathway increased in rat mammary gland in close agreement with the myo-inositol content of milk and diminished to prelactation activities within 24 hours after the onset of involution.

The myo-inositol levels in selected tissues including brain, heart, lung, liver, kidney and intestine were compared for two day prepartum, weanling and pregnant female rats. Data were consistent with increased retention of myo-inositol during development in the rat. Effects of

2-deoxy-D-glucose administration and dietary myo-inositol on fetal tissue myo-inositol were also examined. Potential contributions of diet and biosynthesis of myo-inositol to fetal tissue myo-inositol levels are discussed.

The myo-inositol level of colostrum and milk of five human subjects was highest (2.8 mM) before birth and decreased to 40% of that level five days postpartum, where it remained for at least three weeks. Even after seven months of lactation, the milk of one subject contained 3-4-fold more myo-inositol than all commercial infant formulas analyzed.

INTRODUCTION

Previous studies of D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (synthase, formerly cyclase, EC 5.5.1.4) and L-myo-inositol-1-phosphate phosphatase (phosphatase, EC 3.1.3.25) in mammalian tissue have been largely confined to rat testis (1-4). Although the pathway leading to myo-inositol is assumed to occur in virtually all mammalian cells, though at variable rates, little is known about the existence and regulation of the enzyme system in other tissues, e.g., brain (5). In slice experiments with radioactive precursors, Hauser and Finelli (6) demonstrated incorporation of ^{14}C from glucose into myo-inositol in liver, kidney and brain.

In studies of a number of animal species, Nixon (7) confirmed an older observation by Offergeld (8) that the myo-inositol content of fetal blood consistently exceeded that of the maternal blood by nearly ten-fold, suggesting a critical function for myo-inositol in development. Studies by Nixon (9) on exteriorized sheep fetuses

provided indirect evidence to support the hypothesis that fetal blood myo-inositol was derived from the fetus, not the placenta or maternal circulation. Furthermore, Andrews et al. (10) found that perfused fetal sheep livers were capable of synthesizing myo-inositol. Since the origin of the fetal and neonatal blood myo-inositol has not been fully established, an investigation of the developmental pattern of the synthase and phosphatase in fetal, neonatal, and maternal rat liver as well as in the mammary gland at various stages in lactogenesis was conducted to provide direct evidence for the ability of these tissues to synthesize myo-inositol and to correlate the enzyme activities with the levels of myo-inositol in plasma and milk. In addition, effects of 2-deoxy-D-glucose administration, the 6-phosphate of which is a strong competitive inhibitor (K_i=20 uM) of the synthase reaction (12), on fetal myo-inositol levels were studied in order to seek further evidence for synthesis as a significant source of fetal myo-inositol.

MATERIALS AND METHODS

Reagents. The following materials were obtained from the indicated sources. Glucose-6-phosphate dipotassium salt, nicotinamide adenine dinucleotide (NAD^+), and ascorbic acid from Sigma Chemical Co.; L-chiro-inositol-3-phosphate and dithiothreitol (DTT) from Calbiochem; ethylenediaminetetraacetic acid (EDTA) from Matheson, Coleman & Bell; myo-inositol and 2-deoxy-D-glucose from Nutritional Biochemicals; α-methyl mannoside from General Biochemicals, Inc.; 3% OV-1 (w/w) on Chromosorb W (100-200 mesh) from Applied Science Laboratories, Inc.; and trimethylchlorosilane and hexamethyldisilazane from Pierce Chemical Co.

Animals. Pregnant rats of the Holtzman strain (Madison, Wisconsin) weighing 240 g were used in the study. Litters were adjusted to 7 or 8 pups, and all dams were fed a commercial pellet diet and water ad libitum.

For experiments with 2-deoxy-D-glucose (2DG), fetuses from dams fed a myo-inositol supplemented or deficient diet as described elsewhere (see Chapter III) were sampled at three days pre-partum. Each dam was injected intraperitoneally with 100 mg/kg body weight 2-deoxy-D-glucose or saline daily for two days prior to sampling. Sampling occurred 24 hours after the last injection. Fetal age was estimated from the sperm positive date supplied by the Holtzman Company. Fetuses were removed from the dam under ether anesthesia and liver, brain and kidney samples were subsequently removed and stored at -80° C. Blood samples were collected by cardiac puncture with heparinized syringe or hematocrit tubes, cells removed by centrifugation and the resultant plasma pooled from each litter and stored at -80° C.

Enzyme Preparation: Synthase and Phosphatase. As noted by earlier workers (1, 11), extracts from mammalian tissues normally contain very active phosphatases and phosphoglucose isomerase which compete for the substrate, D-glucose-6-phosphate, causing serious difficulties in the measurement of total synthase activity. These problems can be partially obviated by controlled heat treatment, and synthase can be conveniently assayed by the periodate oxidation procedure of Barnett et al. (12).

Synthase was prepared according to the method of Barnett et al. (12) with the following modifications. The heat treatment to inactivate nonspecific phosphatases and phosphoglucoseisomerase was carried out immediately after extraction in a Potter-Elvehjem homogenizer and

prior to the centrifugation at 105,000 xg. The inactivation was extended to 10 minutes at 60° C, since this was shown to provide a higher yield of total enzyme activity. Ammonium sulfate fractions from 0 to 40% of saturation were used in order to recover as much of the enzyme as possible. Dialyzates were centrifuged at 14,500 xg for 15 minutes to remove any insoluble protein appearing during dialysis. The samples were either frozen at -80° C for up to one week or assayed at once, both procedures giving identical results.

The phosphatase was isolated from the supernatant fraction of the synthase preparations by adjusting from 40% to 60% of saturation with ammonium sulfate. The precipitated proteins were dialyzed overnight at 4° C against four liters of 50 mM Tris-acetate, pH 7.5, and 1 mM β-mercaptoethanol.

Synthase Assay. Synthase was assayed according to Barnett et al. (12) with minor alterations. These variations included a change to 10 mM EDTA to minimize unremoved phosphatase activity, 14 mM ammonium acetate, and the addition of 1 mM dithiothreitol. Samples were assayed for free phosphate as described below. One unit of synthase is defined as 1 umole of L-myo-inositol-1-phosphate produced per hour at 37° C.

L-myo-Inositol-1-Phosphate Phosphatase Assay. The phosphatase procedure followed was essentially that of Eisenberg (3). The reaction mixture contained 40 mM Tris-acetate, pH 7.4; 100 mM KCl, 3 mM $MgCl_2 \cdot 6 H_2O$, 0.2 mM L-myo-inositol-1-phosphate or L-chiro-inositol-3-phosphate and 0.1 ml enzyme extract in an assay volume of 0.5 ml and was incubated at 37° C for 30 minutes. The reaction was terminated by adding 0.25 ml of 20% TCA, and the protein was removed by centrifugation. A 0.5 ml aliquot was analyzed directly for free phosphate as indicated below. A

blank in which TCA was added prior to the addition of the enzyme extract was used to account for free phosphate and that released by action of the TCA. One unit of enzyme activity is defined a 1 umole of phosphate released per hour at 37° C.

Determination of Phosphate. Phosphate was determined using the method of Ames (13) with minor variations. Phosphate color reagent (2.1 ml) containing six parts 0.42% ammonium molybdate·4 H_2O in 1.0 N H_2SO_4 and one part 10% (w/v) ascorbic acid were added to 0.9 ml of the sample to be assayed. The color reaction was incubated at 45° C for 20 minutes and absorbance was measured at 700 nm in a Gilford Model 300 spectrophotometer.

Protein Determination. Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as a standard.

Gas Chromatography of myo-Inositol. Plasma and tissue samples were deproteinized according to Somogyi (15). The suspension was centrifuged to remove the precipitated protein and α-methyl mannoside was added to an aliquot of the supernatant as an internal standard. The sample was mixed thoroughly and taken to dryness on a rotary evaporator. Samples were then analyzed for myo-inositol according to Wells et al. (16).

Mammary Tissue. For analysis of mammary gland free myo-inositol levels, tissue was removed, minced, and soaked for 60 minutes at 0° C in an oxytocin:saline solution (10 IU of oxytocin per liter of 0.9% NaCl) to release contaminating milk.

Collection of Milk. In the morning, lactating rats were lightly anesthetized with sodium pentabarbital (60 mg/kg) and injected with 0.15 ml of an oxytocin solution (10 IU/ml). Milk was collected by a

gentle suction apparatus employing a water aspirator. At various periods pre- and postpartum, milk was also collected from five human volunteers ranging in age from 22 to 33 years.

Preparation of L-myo-Inositol-1-Phosphate. We are indebted to Dr. Laurens Anderson, University of Wisconsin, for a generous gift of a mixture of approximately 67.8% L-myo-inositol-1-phosphate and 32.2% myo-inositol-2-phosphate as determined by gas-liquid chromatography of the fully trimethylsilylated compounds. The retention times were further verified by authentic standards of the monophosphate esters generously provided by Dr. C.E. Ballou, University of California, Berkeley. For further supplies of the substrate which is unavailable commercially, we carried out large-scale conversion of D-glucose-6-phosphate to L-myo-inositol-1-phosphate with partially purified synthase from rat testis (30-40% ammonium sulfate fraction). A typical reaction contained 50 mM Tris-acetate, pH 8.0, 10 mM sodium EDTA, 14 mM ammonium acetate, 5 mM potassium glucose-6-phosphate, 0.1 mM NAD^+, 1 mM DTT, 20 mg of a commercial antibiotic preparation, and 10.3 units of synthase, in a final volume of 500 ml. After 169 hours the reaction was stopped by boiling, and the protein was removed by centrifugation. A yield of approximately 13% of L-myo-inositol-1-phosphate was achieved under these conditions. The product was recovered and separated from D-glucose-6-phosphate by passage over a BioRad AB 1x8, 200-400 mesh column (1.5 x 60 cm) in the formate form and equilibrated with 0.1 M ammonium formate, 0.02 M sodium borate, pH 9.5, at a flow rate of 0.4 ml/minute (17). The sugar phosphates were eluted with a gradient consisting of two chambers of 0.1 M ammonium formate, 0.02 M sodium borate, pH 8.5 (150 ml each) and a third chamber of 0.625 M ammonium formate in 0.02 M

sodium borate, pH 8.5. Fractions of 10 ml were collected and the myo-inositol-1-phosphate peak which preceded the glucose-6-phosphate from the column was found routinely in fractions 35-49 and glucose-6-phosphate at tubes 50-55. Glucose-6-phosphate was detected spectrophotometrically (18). Tubes containing the myo-inositol-1-phosphate were pooled and treated with 0.1 volume of saturated $Ba(OH)_2$; the barium salt of the phosphate ester was precipitated by the addition of four volumes of 95% ethanol and isolated by standard techniques (19). To prepare stock solutions of the inositol phosphate, barium was exchanged with hydrogen by Dowex 50 (H^+) and contaminating boric acid evaporated as methyl borate after two to three successive additions of methanol. The product displayed an identical retention time with that of authentic L-myo-inositol-1-phosphate as the trimethylsilylated derivative on a 1.8 m by 6 mm column of Chromosorb W coated with 3% OV-1 at 230° C (20) and was quantified by the periodate phosphate elimination reaction (12).

RESULTS

myo-Inositol Content of Selected Tissues in the Weanling, Fetal and Pregnant Rat. Table 1 displays free myo-inositol contents of selected tissues in the weanling, fetal and pregnant rat. Generally, the fetal-maternal tissue myo-inositol levels reflected the large (7-8-fold) fetal-maternal plasma myo-inositol gradient. Fetal tissue myo-inositol contents were 2- to 4-fold higher than that of the corresponding maternal tissue (significance as seen in Table 1); however, several tissues, notably brain and lung, showed no significant differences in myo-inositol content.

TABLE 1

myo-Inositol Content of Selected Tissues in the Weanling, Fetal and Pregnant Rat

Tissue	Animals[a,b]		
	Pregnant Female	Fetus	Weanling
Liver	0.159 ± 0.018[4]	0.449 ± 0.091[3]	0.235 ± 0.064
Brain	5.04 ± 0.57	4.51 ± 0.66	3.52 ± 0.46[1]
Lung	0.816 ± 0.166	0.799 ± 0.043	0.872 ± 0.155
Kidney	5.83 ± 0.49[5]	1.58 ± 0.06[5]	6.94 ± 0.35[1]
Intestine (small)	0.902 ± 0.258[1]	1.50 ± 0.16	1.26 ± 0.10
Heart	0.306 ± 0.034[3]	0.524 ± 0.053[3]	0.284 ± 0.013
Mammary	0.437 ± 0.016	-----	-----
Ovaries	0.754 ± 0.184	-----	-----
Testes	-----	-----	5.03 ± 0.22
Placenta	0.531 ± 0.149	-----	-----
Carcass (-tissues)	-----	0.857 ± 0.029	-----
Plasma	82.3 ± 2.8	614.5 ± 44.7[5]	86.2 ± 24.0

[a]Values are the mean ± S.D. for 4 dams, 4 weanlings or 4 pools of fetuses (one pool from each dam) expressed as umoles of myo-inositol/ g fresh weight tissue or uM myo-inositol for plasma. Dams and weanlings (21 days of age) were maintained on a commericial pellet diet and water ad libitum. Fetuses were 2 days prepartum and the dams their corresponding mothers. Determination of myo-inositol was as described in the Methods section

[b]Statistics - Superscripts in each column refer to the following comparisons: 1) pregnant female column - female vs. fetus; 2) fetal column - weanling vs. fetus; 3) weanling column - female vs. weanling. The following numbers refer to the level of significance observed using the Student's t test: 1. $p<0.05$; 2. $p<0.02$; 3. $p<0.01$; 4. $p<0.005$; 5. $p<0.001$.

When weanling rats were compared with pregnant dams, significant differences were noted for brain ($p<0.025$) and kidney ($p<0.05$) in the tissue levels of myo-inositol. As the rat developed from fetus to weanling, significant changes were observed for liver ($p<0.01$), kidney ($p<0.001$) and heart ($p<0.01$) but not lung, intestine or brain. Other tissues, including mammary, the gonads and placenta, as well as fetal carcass, are listed for comparative purposes.

Tissue-plasma myo-inositol concentration gradients were observed in the fetus (T/P range: 7-fold to 0.7-fold) and the weanling (T/P range: 80-fold to 3-fold) rat. Weanling tissue-plasma differences reflected those observed in the pregnant dam and were generally ≥ 3. Fetal gradients, however, revealed smaller tissue-plasma ratios generally ≤ 3 and were always lower than in the corresponding weanling tissue ratios, respectively.

myo-Inositol Content of Rat Plasma. Figure 1 shows the developmental pattern of the myo-inositol content of rat plasma for pups and dams. In the maternal blood, a small decrease in the level of plasma myo-inositol occurred shortly after parturition which returned to prepartum levels by eight days postpartum. The myo-inositol in the plasma of the fetuses dropped abruptly prior to parturition, but plateaued after birth at concentrations approximately four-fold higher than maternal blood which persisted for nearly 16 days postpartum.

myo-Inositol Content of Rat Milk and Mammary Gland. A comparison of the myo-inositol content of rat milk and the free myo-inositol content of rat mammary gland is shown in Figure 2. A three-fold increase in the milk myo-inositol content was observed during the developmental periods examined, and a similar increase was noted in the mammary tissue.

Figure 1. myo-Inositol Content of Rat Pup Plasma (◇) and Maternal Plasma (◆) During Development. Days indicated by either a minus sign or without a sign refer to days prepartum or days postpartum, respectively. Each point represents the mean ± S.D. of 4 pools of 8 pups and 4 dams. Analytical procedures are as described under Methods.

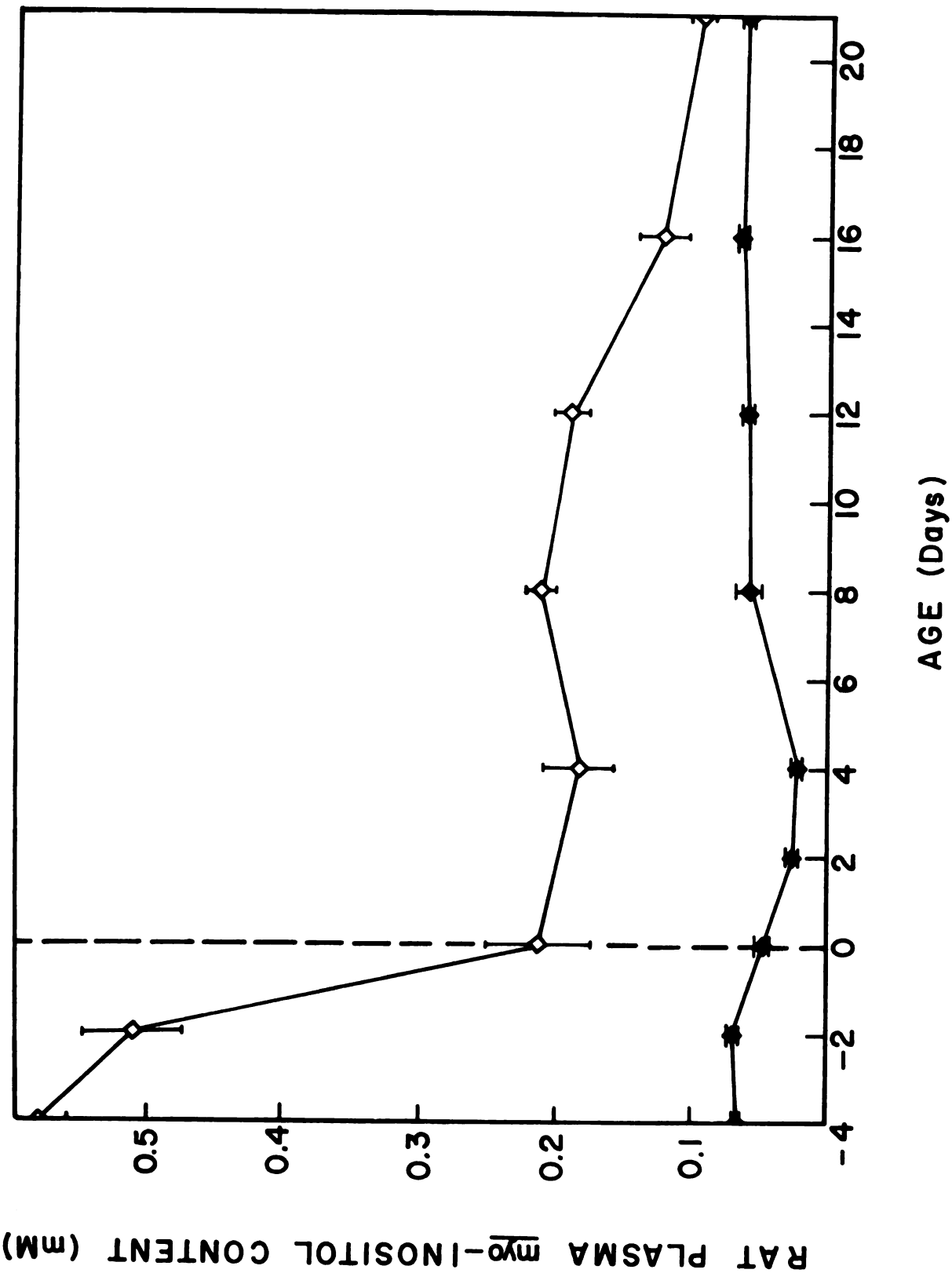
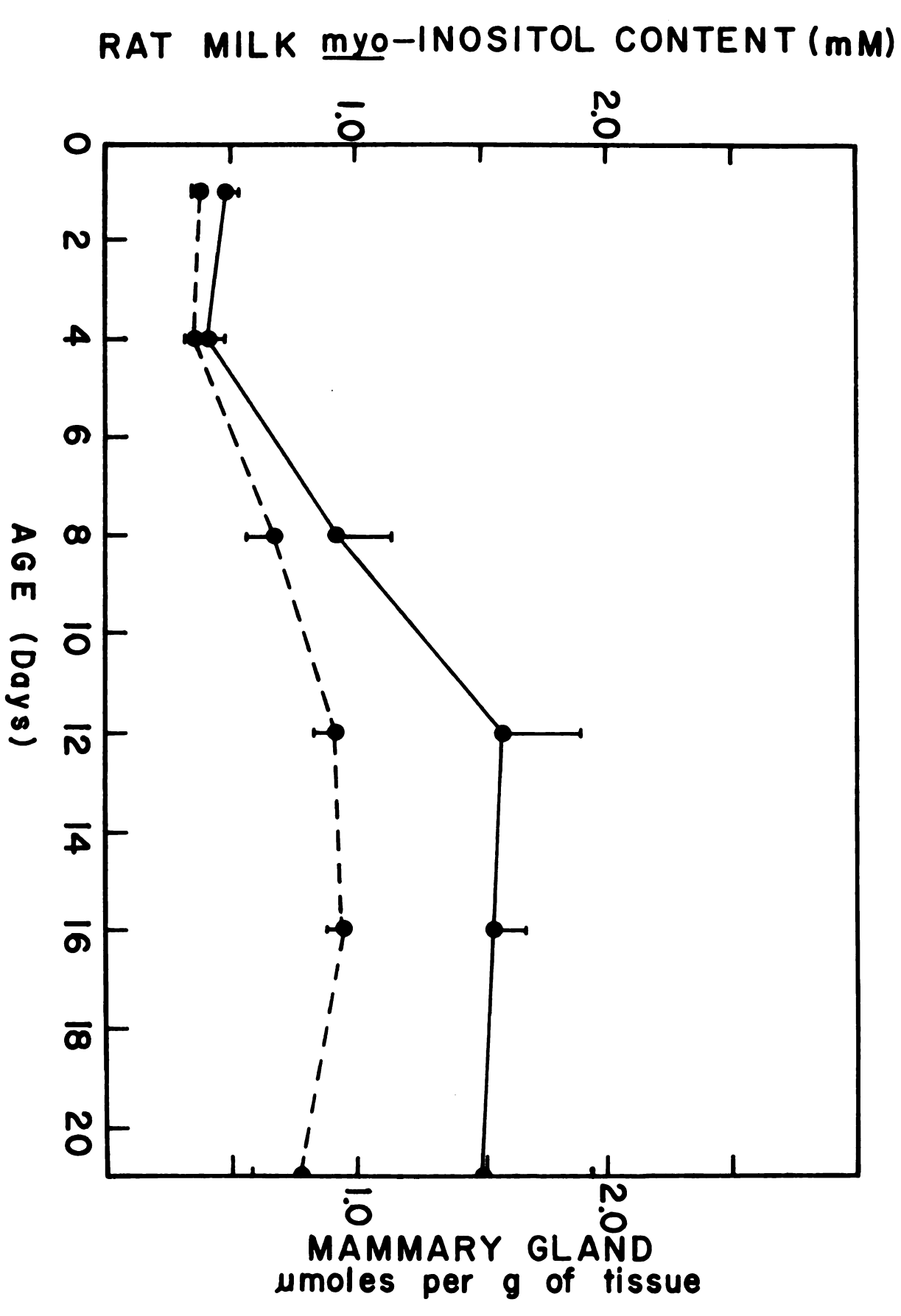

RAT PLASMA myo-INOSITOL CONTENT (mM)
0.5
0.4
0.3
0.2
0.1
-4
-2
0
2
4
6
8
10
12
14
16
18
20
AGE (Days)

Figure 2. myo-Inositol Content of Rat Milk and Mammary Gland During Lactation. All are from samples obtained postpartum. Each value represents the mean ± S.E.M. of 4 animals. Tissue preparation and assay conditions are as described in the Methods section. (———), milk; (- - - -), mammary gland.


RAT MILK myo-INOSITOL CONTENT (mM)
1.0
2.0
0
2
4
6
8
10
12
14
16
18
20
AGE (Days)
1.0
2.0
MAMMARY GLAND
µmoles per g of tissue

A similar observation of increased myo-inositol in mammary glands during lactation was reported by Dawson and Freinkel (21), but separate analysis of milk was not reported for their study.

Human Milk myo-Inositol. The level of free myo-inositol was found to be relatively high in the colostrum and milk of five human subjects (Figure 3), and this was distinctly different than the pattern for rat milk myo-inositol. Within five days postpartum, the mean value was approximately 40% of the highest level and this was followed by a period of relative constancy. One subject was observed at three and seven months postpartum, at which times myo-inositol levels had decreased even further. It is interesting to note, however, that even the lowest levels recorded for human milk in this study are several-fold higher than the values we have determined for commercially available formulas which ranged between 0.14 and 0.21 mM.

Developmental Pattern of Synthase. The highest liver synthase activity was observed in fetal rats. Thereafter, a decline occurred reaching adult levels by day 12 postpartum (Figure 4). As a comparison, the livers of four pools of three fetuses, two-day prepartum, contained over 48% of the total body synthase although the liver represents only 7.8% of the body weight. Prior to parturition, rat mammary gland synthase activity was low and similar to that of adult liver and placenta, but rose during lactation in an inverse relationship with that of newborn rat liver. In either tissue, the activity of the synthase correlated well with the level of free myo-inositol in fetal plasma or rat milk. Similarly, it appears that a slight decrease in maternal liver cyclase activity followed parturition and was accompanied by a parallel decrease in maternal blood myo-inositol level. The synthase activity of

Figure 3. Developmental Changes in Human Milk *myo*-Inositol Levels. Each point represents the mean ± S.E.M. of 5 subjects except for the final two points which are samples from only one of the five subjects.

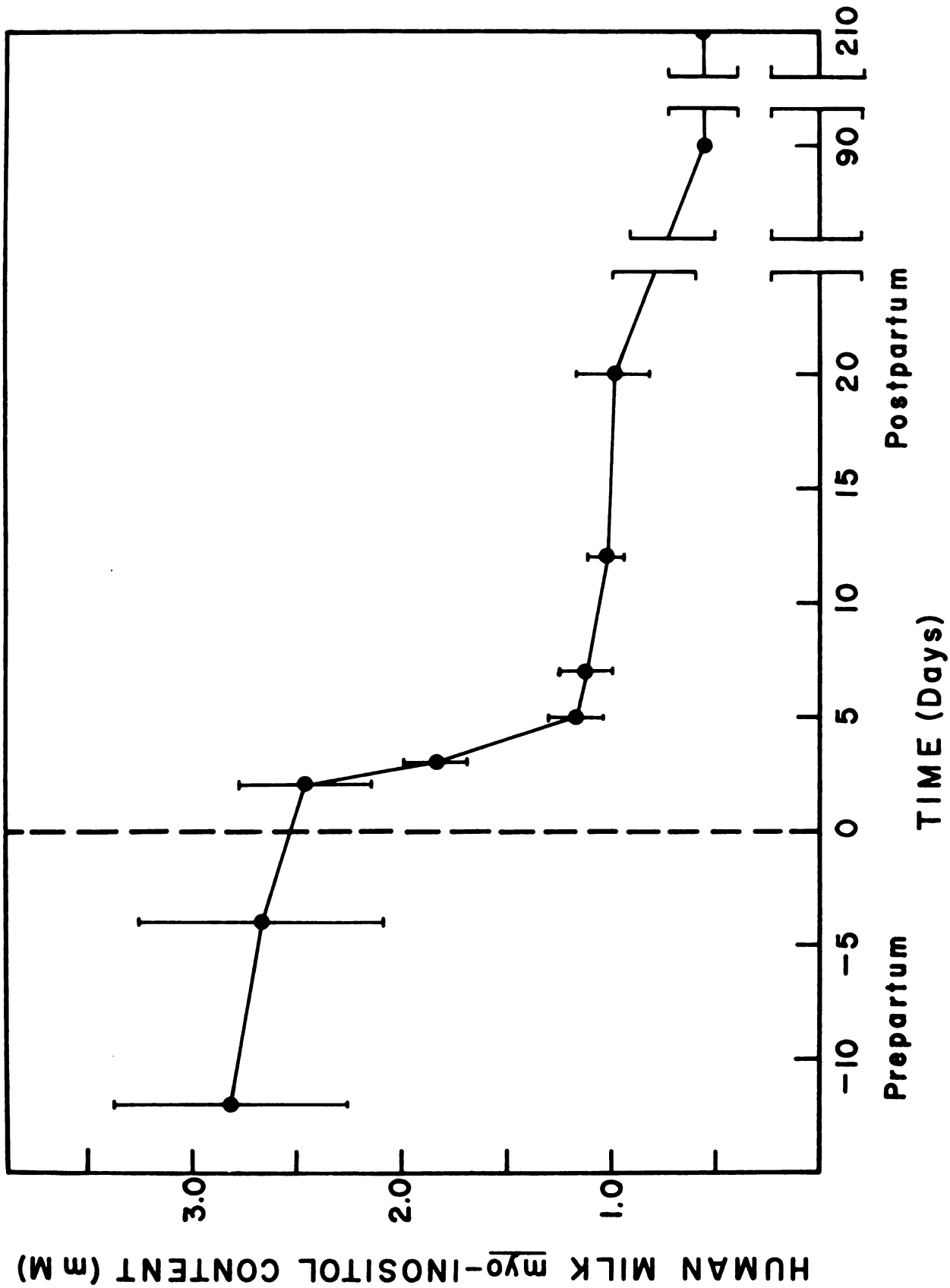
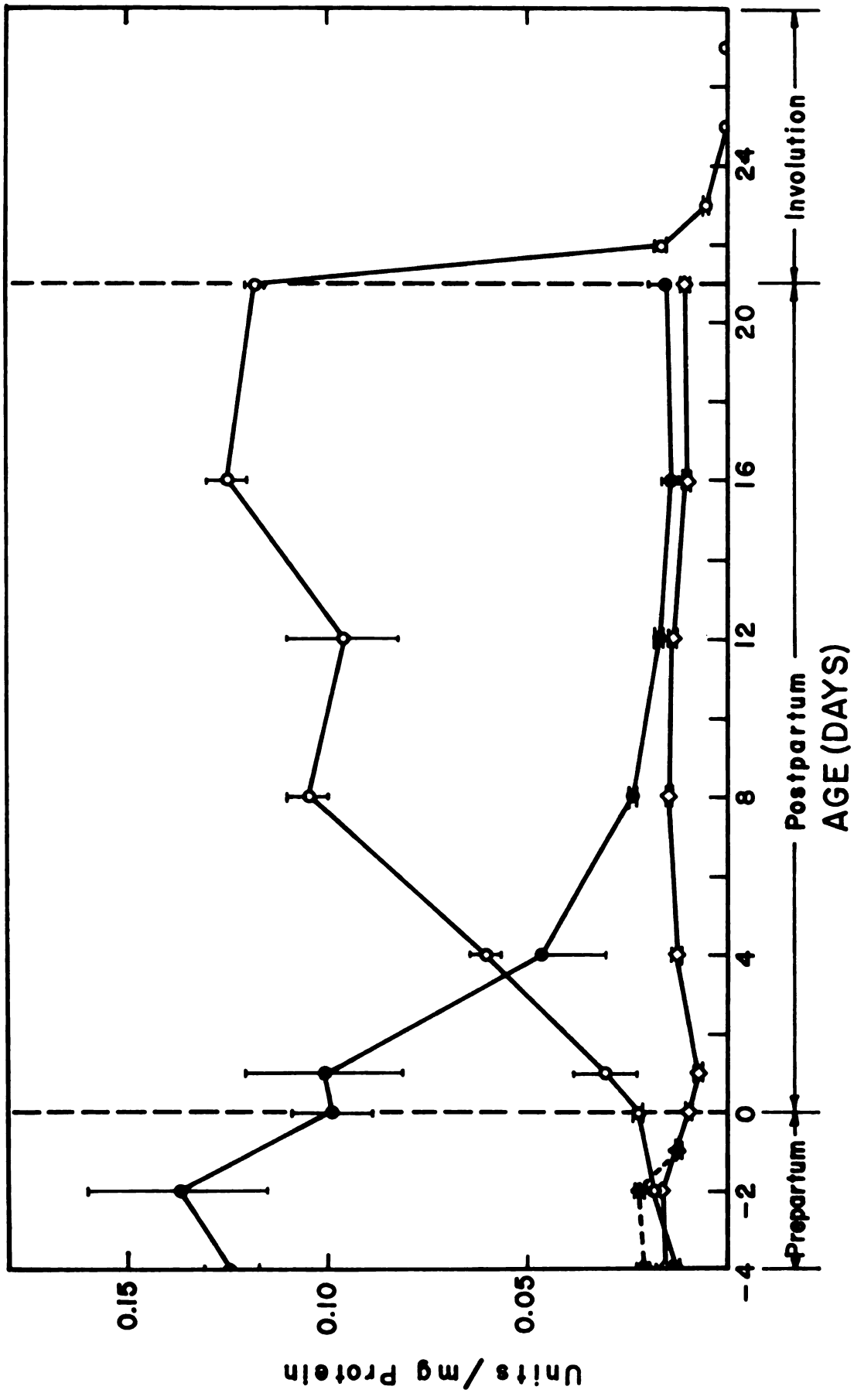

HUMAN MILK myo-INOSITOL CONTENT (m M)
3.0
2.0
1.0
-10
-5
0
5
10
15
20
90
210
Prepartum
Postpartum
TIME (Days)

Figure 4. Developmental Changes in Activity of D-Glucose-6-Phosphate: L-myo-Inositol-1-Phosphate Synthase of Fetal and Neonatal Rat Liver (●), Maternal Liver (◇), Placenta (◆), and Mammary Gland (○). Each value represents the mean ± S.D. for 4 pools of 8 pups, 4 pools of 8 placentae, or 4 dams. Tissue preparation and assay conditions are as described in the Methods section.


Units / mg Protein
0.15
0.10
0.05
-4
-2
0
4
8
12
16
20
24
Prepartum
Postpartum
Involution
AGE (DAYS)

the mammary tissue diminished to prelactation levels within 24 hours after the onset of involution.

Developmental Pattern of the Phosphatase. Analysis of L-myo-inositol-1-phosphate phosphatase revealed a profile similar to that of the cyclase in both fetal and neonatal liver and in mammary gland (Figure 5). In the newborn liver, the phosphatase diminished more rapidly than did the cyclase activity. Another minor variation between the two enzymes can be seen in maternal liver phosphatase shortly after birth of the pups; i.e., an increase was found in maternal liver phosphatase, while a small decrease was found in maternal liver synthase activity (Figure 4). Fetal liver contained a significant amount (26.6%) of the total body phosphatase. Under all conditions studied, however, the activity of the phosphatase was 5- to 10-fold greater than that of the synthase. The patterns for the two enzymes were sufficiently similar to suggest a coordinated response for both enzymes to those factors which regulate their activities.

Effects of Diet and 2-Deoxy-D-Glucose Administration on Fetal Rat Tissue myo-Inositol Content. Administration of 2-deoxy-D-glucose (2DG) intraperitoneally to the pregnant dam reduced the levels of myo-inositol in various tissues in the rat fetus regardless of the availability of dietary myo-inositol to the dam. Table 2 shows the effects of 2DG administration on free myo-inositol levels in plasma, brain, liver and kidney of fetal rats whose corresponding dams were fed a myo-inositol supplemented or deficient diet. A significant ($p<0.01$) dietary effect of myo-inositol on tissue free myo-inositol content was observed only for the kidney. Administration of 2DG to both supplemented and deficient dams resulted in the elimination of the fetal kidney dietary

Figure 5. Developmental Changes in Activity of L-*myo*-Inositol-1-Phosphate Phosphatase of Fetal and Neonatal Liver (●), Maternal Liver (◇), Placenta (◆), and Mammary Gland (○). Each value represents the mean ± S.D. of 4 pools of 8 pups, 4 pools of 8 placentae, or 4 dams. Tissue preparation and assay conditions are as described in the Methods section.

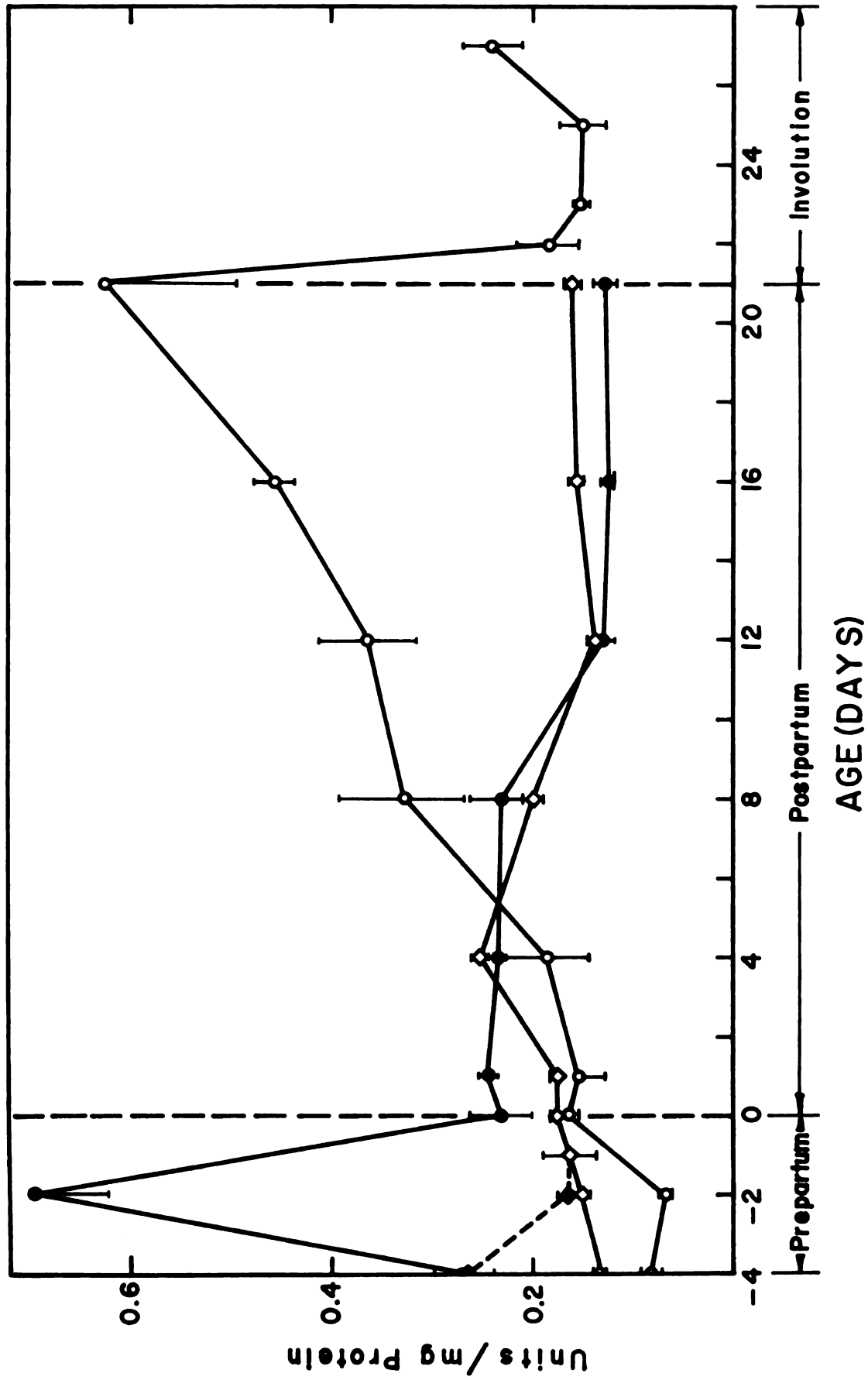

Units / mg Protein
0.6
0.4
0.2
-4
-2
0
4
8
12
16
20
24
Prepartum
Postpartum
Involution
AGE (DAYS)

TABLE 2

EFFECTS OF 2-DEOXY-D-GLUCOSE AND DIET ON FETAL RAT myo-INOSITOL CONTENT OF PLASMA AND SELECTED TISSUES

Tissue	Diet Fed[3]	Treatments [1, 2] Control	2-Deoxy-D-Glucose
maternal plasma	+	115 ± 20	139 ± 34[A]
	-	45.2 ± 22.0[d]	37.4 ± 8.6[e, A]
fetal plasma	+	649 ± 94	430 ± 61[B]
	-	482 ± 102[a]	167 ± 36[e, C]
fetal liver	+	0.592 ± 0.051	0.450 ± 0.034[C]
	-	0.506 ± 0.051[a]	0.221 ± 0.056[d, E]
fetal kidney	+	1.87 ± 0.044	1.03 ± 0.09[F]
	-	1.51 ± 0.11[d]	0.838 ± 0.114[a, E]
fetal brain	+	4.02 ± 0.16	3.75 ± 0.27[A]
	-	3.89 ± 0.52[a]	3.34 ± 0.18[A, a]

[1] Dams were injected with saline (control) or 2-deoxy-D-glucose as given in the Methods section. Statistics: a. not significant ($p > 0.05$), b. $p < 0.05$, c. $p < 0.02$, d. $p < 0.01$, e. $p < 0.005$, f. $p < 0.001$; Upper case letters refer to comparisons between control and 2-doexy-D-glucose groups; lower case letters refer to comparisons within control or 2-deoxy-D-glucose groups. Statistical evaluations were made using the Student's two-tailed t test.

[2] Tissue levels of myo-inositol are given as uM for plasma or umoles/g wet weight tissue for tissues. Each value is the mean ± S.D. for four dams or four pools of fetuses (1 pool from 1 dam).

[3] For diet constituents, refer to the Methods section Chapter III. Supplemented diet (+), Deficient diet (-).

difference, whereas significant reductions appeared in fetal plasma ($p<0.005$) and fetal liver ($p<0.01$) content of myo-inositol (see Table 2). Although reductions occurred in fetal plasma and liver upon 2DG administration, this did not significantly affect the fetal dietary source (maternal plasma) of myo-inositol. Comparisons of groups consuming the same diet with or without 2DG administration showed significant ($p<0.05$) decreases in the myo-inositol content of the tissues examined with the single exception of brain. Although the mean values for brain myo-inositol content showed expected changes with all treatments, no significance could be attached to the observations.

DISCUSSION

In this study, tissue and plasma free myo-inositol contents were compared in the two day prepartum fetal and the weanling rat and fetus versus the corresponding dam (Table 1). Fetal rat plasma myo-inositol levels revealed a large gradient (7-8-fold, Table 1, Figure 1) across the placenta as previously described for other mammals (22). The mechanism by which the fetal and newborn rat blood myo-inositol level is maintained and regulated in excess of maternal circulation is not understood in detail. The results of Nixon's (9) studies suggested that the fetus itself could account for the blood myo-inositol when sheep fetuses or their isolated livers were perfused. The present work extends this concept by providing direct evidence for the occurrence and relative activity of the enzymes. Synthase and phosphatase, involved in the biosynthetic pathway of myo-inositol. In fetal liver, this pathway appears to be under developmental control and declines after birth at a relatively rapid rate. This decline in synthetic activity

is paralleled by corresponding decreases in liver, brain (see also Chapter V), and plasma myo-inositol content of the weanling rat. Tissue increases were observed for kidney myo-inositol levels while lung myo-inositol levels remained unchanged, however, corresponding biosynthetic enzyme measurements have not yet been made in those tissues.

Tissue-plasma gradients were observed in the fetal and weanling rat similar to those observed in the adult (21). Dawson and Freinkel (21) have previously proposed a cellular permeability barrier to myo-inositol in adult rat tissues. These data (Table 1) suggest the development of an increasing tissue ability to retain myo-inositol by a change in the cellular permeability barrier within the cell wall as the fetus develops to adulthood. In addition, it suggests that by the weanling stage of development in the rat, most of the tissues studied have attained the adult's ability to retain high levels of intracellular myo-inositol. This observation may prove to be important with regard to myo-inositol depletions and deficiency studies.

Work with 2-deoxy-D-glucose (Table 2) reveals an approximate 60% dependence of the fetus upon endogenous biosynthesis as a source of fetal plasma myo-inositol. This would suggest that both biosynthetic and dietary (via placental transport) sources of myo-inositol can play a significant role in determining the free myo-inositol content of fetal tissues in the rat. In view of tissue retention ability and the minimal 2-deoxy-D-glucose exposure, however, the relative importance of biosynthesis as a major fetal source of myo-inositol may be greater than the data indicates.

Dawson and Freinkel (21) noted that the myo-inositol content of the

rat mammary gland was much higher when the animal was lactating. We have confirmed this observation and correlated the cyclitol level with the enzyme activities responsible for its biosynthesis. These activities vary during development in synchrony with one another suggesting a common mechanism of biosynthetic regulation. The ability of the newborn rat pup to sustain blood myo-inositol in excess of that found in maternal blood can be attributed to the high myo-inositol content of rat milk which, as in the case of human milk (Figure 3), is one of the richest sources of myo-inositol of animal origin (23). Thus, it would appear that a dietary source (milk) of myo-inositol can appreciably affect the plasma level of myo-inositol in newborn rats. The milk of a number of species have varying myo-inositol levels (24), and we have confirmed that bovine milk (bovine colostrum contains 0.5 mM myo-inositol, whereas bovine milk is approximately 0.1 - 0.2 mM) is one of the poorest milk sources of the polyol. Although there has been no exhaustive study, it is interesting to note that those mammals which give birth to immature young secrete milk with the highest levels of myo-inositol.

During the involution phase of lactation, a striking decrease in myo-inositol synthesizing enzyme activities was observed. The mechanism of mammary gland involution suggested by others (25) may involve the blockage of circulation to the engorged gland inhibiting new enzyme synthesis and simultaneously stimulating the release of lysosomal enzymes. Questions still remain concerning the factors which initiate the increased enzyme activity phase of lactation as well as those regulating liver activity during fetal and postpartum growth and development.

Finally, these studies call attention to the high myo-inositol

levels in human milk and the fact that many formulas are not fortified with myo-inositol. From this, it may be speculated that myo-inositol supplemented formulae should be tested for their efficacy in the growth and rehabilitation of prematurely born infants.

ACKNOWLEDGEMENTS

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CHAPTER II

EFFECTS OF DIETARY myo-INOSITOL ON TISSUE LEVELS OF FREE myo-INOSITOL IN THE RAT

ABSTRACT

The effect of dietary content of myo-inositol on tissue levels of free myo-inositol was examined in a group of selected tissues in the female rat. When compared to commercial stock diet fed controls, a myo-inositol deficient diet succeeded in significantly ($p < 0.05$) reducing levels of free myo-inositol in kidney and heart but not in brain, spleen, liver, intestine or lung. Similarly, a myo-inositol supplemented diet produced significant ($p < 0.05$) elevations in tissue myo-inositol content above that observed for stock diet fed animals in lung, intestine and heart but not in kidney, spleen, brain, mammary gland or liver. Effects of intestinal flora in minimizing dietary deprivation of myo-inositol are discussed.

INTRODUCTION

Conflicting reports (1-11) on the effects of dietary myo-inositol, many regarding its lipotropic function (1-5), have been numerous. These contradictions have resulted in examination of impure diets (11, 12) and intestinal flora (13) as potential, previously unaccounted for exogenous sources of myo-inositol. Endogenous sources such as tissue biosynthesis (12) have also added to the difficulty of interpreting the effects of dietary myo-inositol in tissues. None of the studies previously reported have examined the effects of deficient or supplemented diets on tissue levels of myo-inositol as a significant determinant of

the role(s) of myo-inositol in biological functions.

The purpose of the present study was to examine the effects of dietary myo-inositol supplementation or deprivation on tissue levels of myo-inositol in selected tissues of the rat in order to further evaluate its role in biological systems.

MATERIALS AND METHODS

Reagents. The following materials were obtained from the indicated sources: myo-inositol, vitamin-free casein, α-cellulose and Wesson standard salt mix from Nutritional Biochemicals Corporation; soybean oil from Swift Oil Company; choline chloride from Sigma Chemical Company; vitamin mix without myo-inositol from ICN Pharmaceuticals, Inc.; 3% OV-1 (W/W) on Chromosorb W (100-200 mesh) from Applied Science Laboratories, Inc.; trimethylchlorosilane and hexamethyldisilazane from Pierce Chemical Company and α-methyl-D-mannoside from General Biochemicals, Inc.

Animals and Diets. Female rats of the Holtzman strain (Madison, Wisconsin) weighing 250-300 g were used in this study. All rats had been maintained on a commercial pellet diet[1] and water ad libitum prior to starting the purified dietary regimen. The diets[2] used in this study contained either no added myo-inositol or a level of 0.25%. Gas chromatographic analysis (n=4 for each diet) revealed no detectable free

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[1] Lab-Blox, Allied Mills, Inc., Chicago, Illinois

[2] The basal diet consisted of (% by weight): 63.35% sucrose, 25.00% casein, 4.00% Wesson salt mix, 5.00% soybean oil, 0.10% choline chloride, 1.00% vitamin mix, 1.30% α-cellulose, and 0.25% myo-inositol or sucrose. The Wesson salt mix contained (% by weight): $CaCO_3$, 21.00; $CuSO_4$-$5H_2O$, 0.039; Fe_3PO_4, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2$, 0.009;

myo-inositol (<0.001 mg/100 g diet) in the deficient diet or 241.6 ± 5.7 mg/100 g (mean ± S.D.) diet in the supplemented diet (14). Diets were freshly made every two weeks and stored at 4° C. Animals were housed at 20° C in polycarbonate cages with wood shavings, a light cycle of 12 hours (6:00 A.M. - 6:00 P.M.), and food and water were available ad libitum.

Tissue samples were removed under light ether anesthesia, weighed and stored at -80° C. Heparinized blood samples, collected by heart puncture, were centrifuged and the plasma stored at -80° C. Four animals per diet were sampled after 0, 2, 4, 8 and 16 weeks of feeding the diets. Tissues which were examined included heart, lung, brain, liver, kidney small intestine, mammary, spleen, and pancreas.

Tissue and Fluid myo-Inositol Content. Free myo-inositol content of tissues and plasma was measured by gas-liquid chromatography (14). Intestinal samples were cleaned of their contents prior to analysis by saline irrigation and slitting to expose the lumenal surface.

RESULTS

No significant differences were noted in body weight gains or losses during the course of the experiment. In addition, no unusual

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KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.500; NaF, 0.057, and $Ca_3(PO_4)_2$, 14.900. The vitamin fortification mixture furnished the following in mg or I.U. per 100 g of diet: α-tocopherol, 5.0; ascorbic acid, 45.0, choline chloride, 75.0; menaquinone, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 10.; pyridoxine-HCl, 1.0; Ca-pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin B-12, 0.0014; vitamin A (as the acetate), 900 I.U., and vitamin D_3, 100 I.U.

physical characteristics previously associated with myo-inositol deprivation, such as spectacle eye in rats (8) or alopecia in mice (15) and rats (13), were observed in the animals fed the deficient diet.

Effects of Dietary myo-Inositol on Free Pool myo-Inositol in Selected Tissues. Dietary effects of myo-inositol on the free myo-inositol levels of a variety of tissues in the female rat were examined. Figures 1 and 2 show the effects of feeding a myo-inositol supplemented and deficient diet for a period of up to 16 weeks, on heart, liver, lung, brain, pancreas, spleen, mammary gland, kidney, intestine, and plasma myo-inositol content. Tissue myo-inositol content was generally elevated for supplemented animals above that of the deprived animals. Plasma myo-inositol differences (Figure 1A) were significant ($p < 0.05$) when comparing animals fed the two synthetic diets or one of the synthetic diets and the stock diet throughout the time course of the experiment. None of the tissues observed, however, could be said to be directly responsive to the plasma levels of myo-inositol for either the supplemented or deprived animals. Liver tissue (Figure 1A) showed significant ($p < 0.01$) differences between the supplemented and deficient diet fed animals at eight weeks on the diet, however, significant changes were not observed for either of the diets with respect to the stock diet fed controls. Heart and lung tissue (Figure 1B) maintained consistently significant ($p < 0.005$ and $p < 0.05$, respectively) differences in myo-inositol for supplemented versus deprived animals during the course of the experiment. Significant changes in myo-inositol content ($p < 0.05$) were noted in lung tissue of supplemented animals with respect to stock diet fed controls for the duration of the experiment.

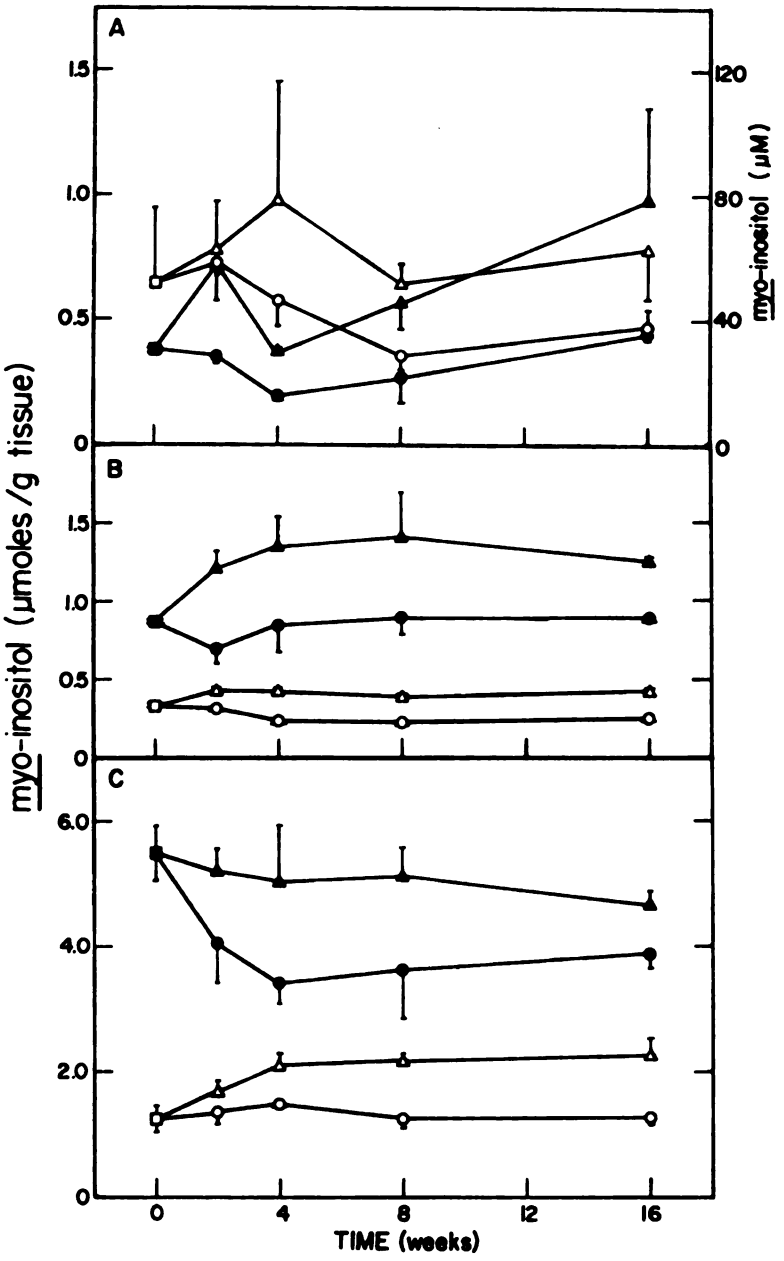

Figure 1. Free myo-Inositol Content of Plasma, Liver, Lung, Heart, Kidney and Intestine in Female Rats Fed a myo-Inositol Supplemented or Deficient Diet. Supplemented animals are represented by the triangular symbols, deprived animals by the circular symbols. Each value is the mean ± S.D. for 4 animals. In A) plasma is represented by the solid symbols, hepatic tissue by the open symbols. In B) lung is represented by the solid symbols, cardiac tissue by the open symbols. In C) kidney is represented by the solid symbols, intestinal tissue by the open symbols. Square symbols represent animals fed the stock diet.

Similar differences were not observed, however, for deprived animals. Renal tissue (Figure 1C) demonstrated significant ($p<0.05$) differences in myo-inositol content between the two groups throughout the regimen; with the deprived animals having levels significantly below ($p<0.05$) that of the stock diet controls. Levels for supplemented animals were not different ($p<0.05$) from the stock diet fed animals. Mean differences were observed for the myo-inositol content of the small intestine (Figure 1C) throughout the time course, becoming significant ($p<0.02$) by four weeks on the diet. Significant differences in intestinal myo-inositol were observed for supplemented animals when compared to stock fed controls after four weeks of diet feeding, however, at no time was this observed for deprived animals. Splenic tissue (Figure 2A) revealed a dietary response pattern of myo-inositol similar to the kidney showing a decrease in the levels in the deprived animals below levels observed for the stock diet fed animals shortly after initiation of the diet regimen. However, significant differences ($p<0.05$) in myo-inositol levels of the spleen between supplemented and deprived animals were observed only between four and eight weeks on the diets. Pancreas free myo-inositol levels (Figure 2A) showed no significant differences between the supplemented and deprived animals, with the exception of levels observed at eight weeks on the diet ($p<0.05$). The level of myo-inositol in the pancreas of stock diet fed animals was not determined. The increase in the myo-inositol content of pancreas from eight to 16 weeks might be due to plasma elevations during that period for both groups, however, the observation was not universal for all tissues examined. Brain free myo-inositol was not significantly affected by dietary myo-inositol. After two weeks of feeding, however, mean differences

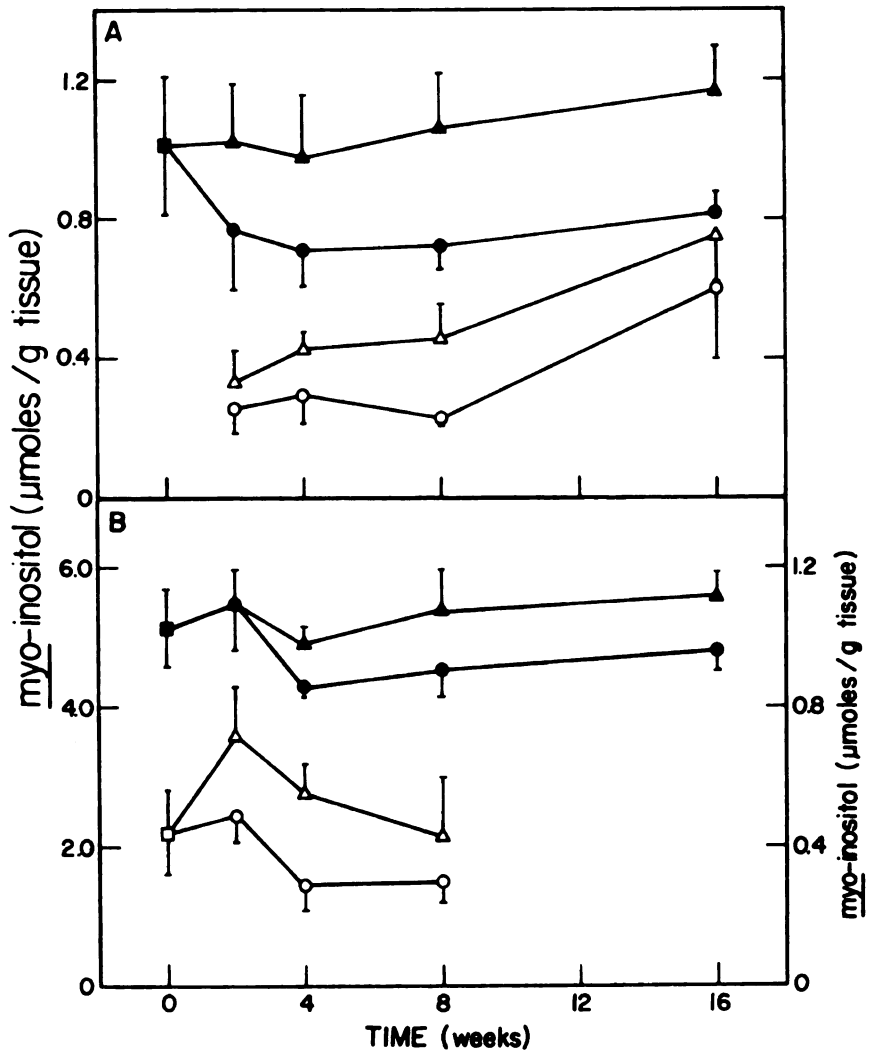

Figure 2. Free myo-Inositol Content of Spleen, Pancreas, Brain and Mammary Gland in Female Rats Fed a myo-Inositol Supplemented or Deficient Diet. Supplemented animals are represented by the triangular symbols, deprived animals by the circular symbols. Each value represents the mean ± S.D. for 4 animals. In A) splenic tissue is represented by the solid symbols, pancreatic tissue by the open symbols. In B) whole brain tissue is represented by the solid symbols, mammary tissue by the open symbols. Square symbols represent animals fed the stock diet. Stock fed pancreatic and 16 week supplemented and deficient diet fed values for myo-inositol were not determined.

between supplemented and deprived animals, though not significant, were observed through 16 weeks on the diet (Figure 2B). Mammary gland (Figure 2B) showed wide variation in myo-inositol content in response to the diets throughout the time course, the significance of which has not been determined. Levels of mammary gland myo-inositol were not determined at 16 weeks for animals fed either of the purified diets.

DISCUSSION

Studies regarding the role of dietary myo-inositol in controlling tissue and body fluid myo-inositol levels are lacking. A significant amount of the dietary work concerning myo-inositol has been centered on its role as a lipotropic agent (1 - 5) without the benefit of diet or tissue analysis for measurements of intake or affects on tissue levels of myo-inositol. This fact has resulted in some difficulty in the interpretation of data and the subsequent evaluation of the role myo-inositol plays in diet (6 - 11). The purpose of this work was to examine the effects of dietary myo-inositol on tissue and plasma free myo-inositol levels and the use of diet as an aid in elucidating the metabolic functions of myo-inositol.

None of the reported symptomatic responses (8, 13, 15) to myo-inositol deprivation were observed during this study. This might be explained by the lack of a sufficient depletion of tissue myo-inositol for most tissues during exposure to the deficient diet.

Two effects are reflected by the majority of the tissues examined with regard to the effects of dietary myo-inositol on tissue levels of myo-inositol. While the deficient diet affected kidney, heart and to a

small extent, brain and spleen, by lowering tissue myo-inositol below stock diet controls, it did not affect the other tissues examined. At the same time, the supplemented diet stimulated myo-inositol levels in intestine, lung, heart and plasma above the stock diet controls while showing no significant effects on kidney, spleen, and brain. Eisenberg's work (16) on synthase activity in several of the tissues examined here would suggest a strong effect of diet on cardiac tissue while almost no dietary response would be expected in testicular tissue, a fact which has been verified (Chapter III, 17). The data presented here might be explained in part by tissue/plasma myo-inositol ratios on the basis of tissue differences in the ability to retain myo-inositol (Chapter I, 18).

Earlier studies reported conflicting data (6 - 11) resulting in difficulty in evaluating myo-inositol dietary effects. Although precautions have been taken with dietary constituents to avoid contamination, accompanied by diet analysis to show the availability of myo-inositol in the diets, the inability of the deficiency diet to reduce levels of myo-inositol in certain tissues might be explained by the presence of microorganisms in the gastrointestinal tract which are known to contribute an exogenous source of myo-inositol to the rat (13). Evidence presented here suggests that for the rat, myo-inositol deprivation symptomology is not observed in the presence of intestinal flora (i.e. the absence of antibacterial drugs). The importance of the intestinal flora as a contributor of exogenous myo-inositol via intestinal absorption or coprophagy is also supported by the absence of a myo-inositol deprived, lactation-induced fatty liver in dams fed a deficient diet containing no antibacterial drugs (Chapter IV). However,

in the presence of gastrointestinal bacteria, effects of dietary myo-inositol were observed in some tissues on tissue levels of myo-inositol and supports the importance of diet as a significant contributor of myo-inositol in tissues.

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CHAPTER III

myo-INOSITOL METABOLISM DURING LACTATION AND DEVELOPMENT IN THE RAT. THE PREVENTION OF LACTATION-INDUCED FATTY LIVER BY DIETARY myo-INOSITOL

ABSTRACT

Effects of dietary myo-inositol deprivation were examined during prenatal and postnatal development and during lactation in the rat. The deficient diet contained no detectable myo-inositol while the supplemented diet contained 0.5% (w/w) myo-inositol at the expense of sucrose. Both diets contained 25% casein, adequate amounts of all known vitamins, choline, and essential fatty acids as well as 0.5% (w/w) phthalylsulfathiazole to depress myo-inositol contribution to the diet by microorganisms. Pregnant rats of the Holtzman strain were fed the respective diets during gestation and lactation and pups were fed the corresponding diet after weaning until three months of age. There were no significant differences in body weight between experimental groups. Supplementation of the diet with myo-inositol increased significantly the levels of myo-inositol in plasma, liver, kidney, and intestine of pups at virtually all ages examined, and increased significantly the levels of myo-inositol in the milk and mammary tissue during lactation. During lactation, the myo-inositol deprived dams developed severe fatty livers (31% w/w) characterized by diminished phosphatidylinositol (50%) and total phospholipid phosphorus (57%) levels as compared with controls. After weaning, the liver lipid content of the myo-inositol deprived dams returned to normal (4.5%). The data suggest that a possible threshold level of free myo-inositol (approximately 0.15 umoles/g

lipid-free tissue) was required to prevent fatty liver in lactating dams under these dietary conditions.

Effects of the deficient diet on fertility were also examined. Based on sperm count and production of offspring, there were no differences between the experimental and control males. Females of both groups showed equal ability to produce offspring.

INTRODUCTION

Metabolic functions for myo-inositol, other than a role in membrane components, are obscure. myo-Inositol is an essential nutrient for microorganisms (1), mammalian cells (2), and for animals under specific dietary conditions (3 - 9). Since 1944, conflicting reports have been published on the role of myo-inositol as a lipotropic agent. Handler (4) and, more recently, Kotaki, et al. (5) have demonstrated that lipid deposition can be prevented in rats by dietary myo-inositol. However, pre-treatment of the animals (5) with a low protein, B vitamin-deficient diet followed by supplementation with B vitamins without choline and myo-inositol was necessary. Other reports showed curative effects of myo-inositol on fatty liver in rats fed a fat-free, high carbohydrate, myo-inositol deficient diet (6). Hegsted, et al. (7, 8) produced an intestinal lipodystrophy in female gerbils by withholding myo-inositol. Recently, Hayashi, et al. (9) demonstrated fatty liver formation in rats in the presence of a sulfa drug when fed a myo-inositol deficient diet containing hydrogenated cottonseed oil but not for one containing natural cottonseed oil.

The purpose of this study was to examine the effect of a myo-inositol deprivation in pregnant and lactating rats on the myo-inositol

metabolism in fetal and postnatal offspring. During the course of the study, a lactation-induced fatty liver was discovered that was prevented by dietary supplements of myo-inositol or by termination of lactation.

MATERIALS AND METHODS

Animals and Diets. Female rats of the Holtzman strain[1] weighing 250-300 g were used in all experiments. On the seventh day of gestation, the pregnant rats were divided randomly into two equal groups and fed purified diets with or without myo-inositol for the specified periods. The diets[2] used in this study were patterned after those of Hayashi et al. (9) and were fed ad libitum. Analysis of the mixed diet showed no detectable (<0.001 mg/100 g diet) free myo-inositol in the deficient diet and 504.2 ± 28.4 mg (mean $\pm$ S.D., n=4) per 100 g of diet free myo-inositol in the control diet (10). A supplemental salt mixture[3] was added to the standard Phillips-Hart salt mixture[4] to bring

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[1]Holtzman Company, Madison, Wisconsin

[2]The following dietary materials were obtained from the indicated sources: vitamin-free casein, α-cellulose and vitamin mix without myo-inositol from ICN Nutritional Biochemicals Corporation, Cleveland, Ohio; Phillips-Hart standard salt mix from Teklad Test Diets, Madison, Wisconsin; soybean oil from Swift Oil Company, St. Louis, Missouri; choline chloride and phthalylsulfathiazole from Sigma Chemical Company, St. Louis, Missouri

[3]The supplemental salt mixture consisted of (% by weight): 6.213% $CuSO_4 \cdot 5\ H_2O$, 0.500% $CoCl_2 \cdot 6\ H_2O$, 14.004% ZnO, 38.248% $MnSO_4 \cdot H_2O$, and 41.035% non-nutritive fiber (cellulose)

[4]The Phillips-Hart salt mixture consisted of (% by weight): 30.00%

the salt content up to accepted levels (11). Phthalylsulfathiazole was employed at the 0.5% (w/w) level to prevent potential contribution of myo-inositol to the diet by intestinal flora. The vitamin mixture[5] incorporated into the diet was of standard content from a commercial source. The major difference between the Hayashi diet (9) and the one used in this study was the incorporation of natural soybean oil rather than hydrogenated cottonseed oil. Diets were freshly made every one to two weeks as needed and stored at 4° C. Neonates were nursed by their respective mothers until 21 days of age at which time they were fully weaned to solid diets and water ad libitum. During the lactational period, however, pups had free access to the solid diet as they desired. Animals were housed at 20° C in polycarbonate cages with wood shavings with a light cycle of 12 hours (6:00 A.M. - 6:00 P.M.).

Fetal ages were estimated from the sperm-positive date supplied by the Holtzman Company and correlation with a mean birth time for each group studied. Gestation for the rat fetus was observed to be

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$CaCO_3$, 7.50% $CaHPO_4 \cdot 2\ H_2O$, 0.005% $CoCl_2 \cdot 6\ H_2O$, 0.003% $CuSO_4 \cdot 5\ H_2O$, 32.2% K_2HPO_4, 2.75% ferric citrate, 10.2% $MgSO_4 \cdot 7\ H_2O$, 0.08% KI, 16.7% NaCl, and 0.025% $ZnCl_2$

[5]The vitamin mix composition was as follows (g/kg): vitamin A ester (palmitate and acetate) concentrate (200,000 units/g), 4.5; vitamin D_3 (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; choline chloride, 75.0, and menaquinone, 2.25. The following vitamins were at levels in mg/kg: p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine-HCl, 1.0; thiamine-HCl, 1.0; calcium pantothenate, 3.0; biotin, 20.0; folic acid, 90.0, and vitamin B_{12}, 1.35

21.5 to 22 days. After birth, the number of pups was adjusted to eight per litter. The sampling of tissue was accomplished as much as possible at the same time of the day and in a random order; i.e., no preference was given to sex or diet in the order of killing. Tissue samples were removed from animals under ether anesthesia, weighed at 4° C, and stored at -80° C. Blood samples were collected in heparinized syringes or hematocrit tubes (by puncture), centrifuged, and the plasma was stored at -80° C. Age 0 was considered the 24 hour period beginning with birth and all other ages were calculated relative to that point. Mothers and/or pups were sampled at -3, -2, 0, +4, +8, +14, +21, +32, +49, and 120 (adult) days of age with respect to the pups' birth. Four maternal samples were taken at each time point. Eight pups of unknown sex were sampled at each point from birth until 21 days. On day 21, four male and four female pups were sampled. After 21 days, three male and three female pups were sampled at each time point. Fetuses from four dams in each group were sampled at pre-birth time points.

Milk Collection and Amniotic Fluid. Milk collection was accomplished as previously cited (12). Amniotic fluid was withdrawn with a syringe prior to removal of the fetus from the uterus.

Tissue and Fluid Content of myo-Inositol. Free myo-inositol content of tissues, amniotic fluid, milk, and plasma was measured by gas-liquid chromatography[6] (13). Lipid-bound myo-inositol in the form

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[6]Materials for gas-liquid chromatography of myo-inositol were as follows: α-methyl-D-mannoside from General Biochemicals, Inc., Chagrin Falls, Ohio; 3% OV-1 (w/w) on Chromosorb W (100-200) mesh from Applied Sciences Laboratories, State College, Pennsylvania; and trimethylcholorosilane and hexamethyldisilazane from Pierce Chemical Company, Rockford, Illinois

of phosphatidylinositol was quantified from a neutral Folch lipid extract of tissue by the procedure of Wells, et al. (10). When determining tissue myo-inositol, identical areas were taken from each of the animals. After day +4, small intestine samples were cleaned of their contents prior to analysis.

Lipid Content of Tissue. Liver lipid content was determined gravimetrically by the method of Folch et al. (14). All organic solvents used in the extractions were freshly redistilled.

Phospholipid Phosphorus Levels. Phospholipid phosphorus in the extracted lipids was measured by the method of Ames (15).

Fatty Acid Quantitation. The fatty acid composition of liver and dietary lipids was determined by gas-liquid chromatography of their corresponding methyl esters[7]. Peaks were identified by comparison of retention time with standards, and composition was expressed as area percent of the total observed peaks on the chromatogram.

Sperm Counts. Sperm counts were made following the technique of Kirton et al. (16).

Statistics. The results were analyzed using the Student's two-tailed t test (17). Significance was acceptable when $p < 0.05$.

RESULTS

Body Weight Profile. No significant differences were observed between the body weights of the myo-inositol supplemented animals versus those fed the deficient diet (Figure 1). (The number of animals (n)

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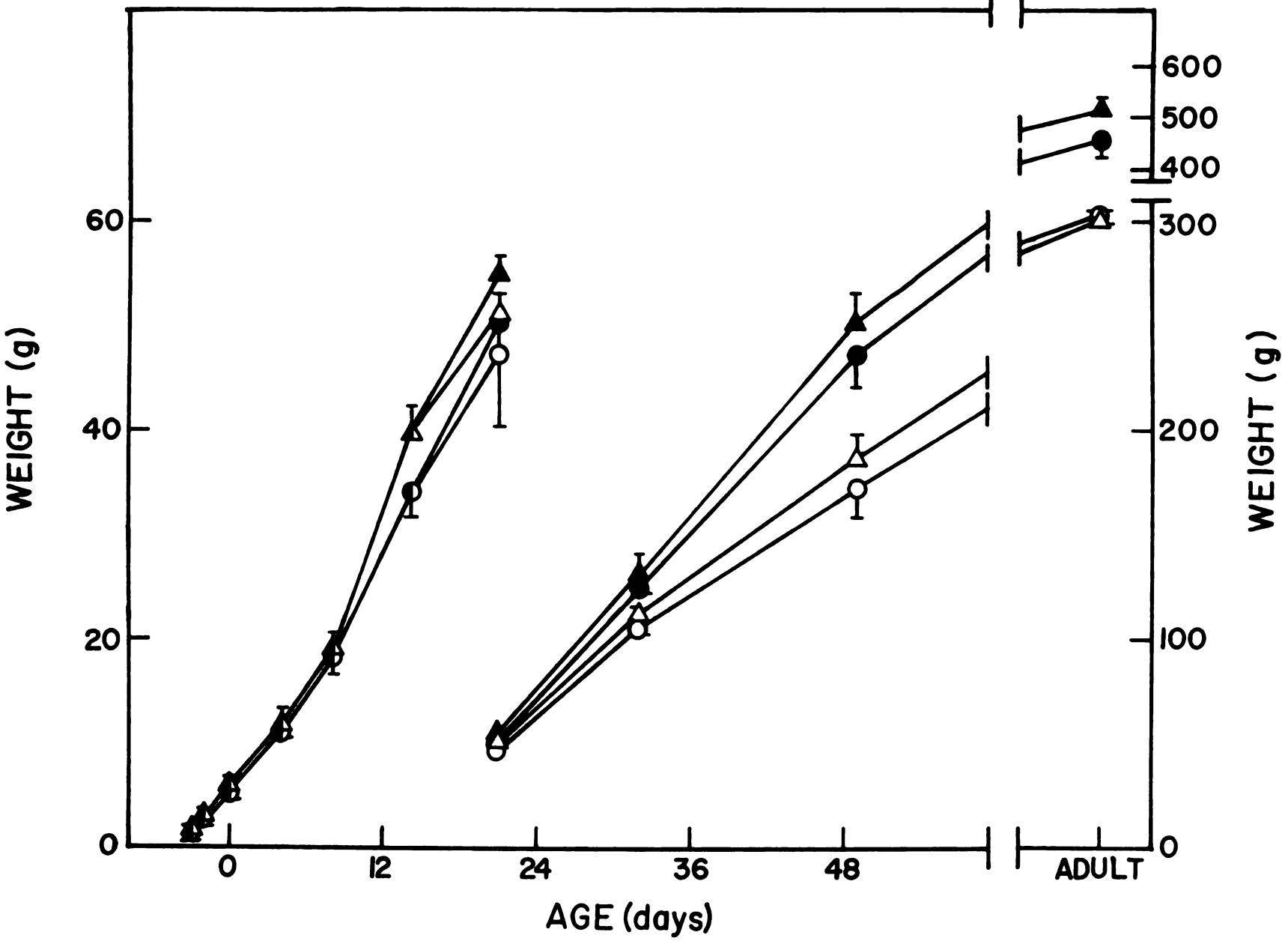
[7]Gas chromatography of the fatty acid methyl esters utilized a 1.8 M by 3mm glass column containing 10% SP 2340 (100-120 on Supelcoport, Bellefonte, Pennsylvania, at 170° C in a Hewlett-Packard Model 402 Gas Chromatograph, Hewlett-Packard Company, Avondale, Pennsylvania

Figure 1. Body Weights of Developing Rats Fed a myo-Inositol Supplemented (▲ , males; △ , females; ◭ , a pool of males and females) or Deficient (● , males; ○ , females; ◐ , a pool of males and females) Diet. Each point represents the mean ± S.D. for n rats. At -3 days, n=35; -2 days, n=35; 0, 4, & 14, n=32; 8, n=20; 21, n=4; 32 & 49, n=12; adult, n=3 rats. Animals 21 days or younger were nursed by dams fed the respective diets. For ages greater than 21 days, animals were fed the same diets as the dams, respectively.

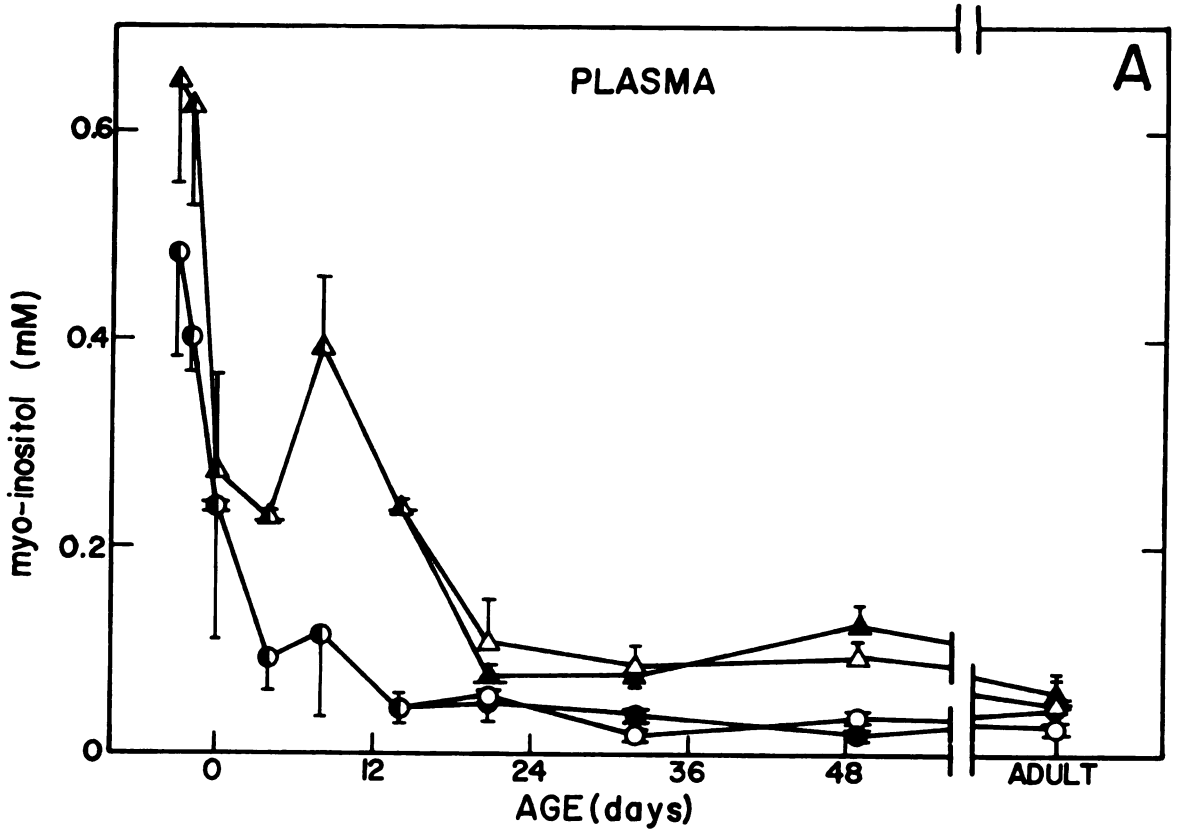

WEIGHT (g)
60
40
20
0
0
12
24
36
48
ADULT
AGE (days)
600
500
400
300
200
100
0
WEIGHT (g)

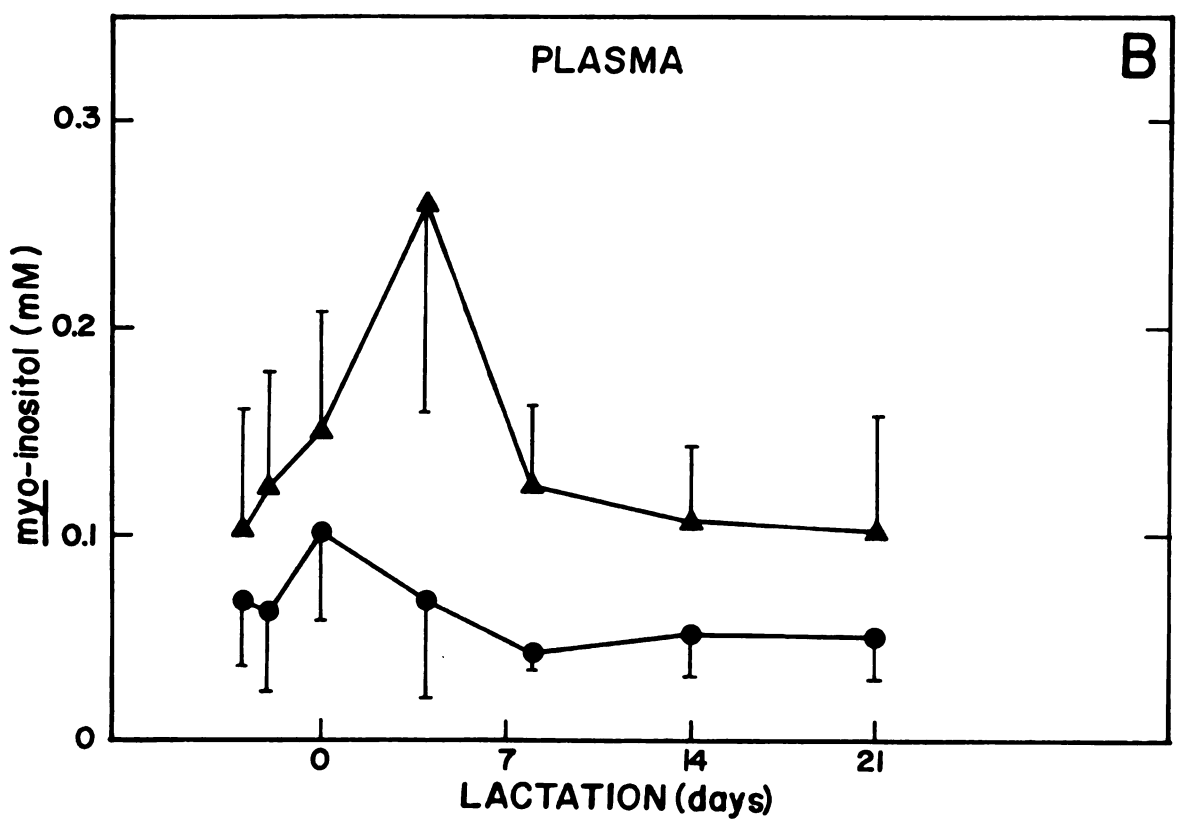
ranged from 8 to 35 for animals up to 120 days of age; for adults (120 days of age), n=3.). However, the mean weight in each group for the supplemented animals was always greater than that of the deficient group. No unusual physical characteristics such as cataracts, alopecia, or deformities were noted in animals of either group.

Amniotic Fluid Free myo-Inositol Levels. Although the mean for the supplemented fetuses was higher than that for the deprived ones in every case, the amniotic fluid myo-inositol levels showed no statistically significant differences. Values observed for amniotic fluid myo-inositol (mean $\pm$ S.D., n=4) in dams fed the deficient diet were 0.215 ± 0.043 mM at -3 days, 0.295 ± 0.024 mM at -2 days, and one value of 0.252 mM at approximately eight hours prior to birth. Supplemented animals showed levels of 0.307 ± 0.043 mM at -3 days, 0.443 ± 0.131 mM at -2 days, and one value of 0.425 mM at approximately eight hours prior to birth.

Plasma Free myo-Inositol Levels. Figure 2A indicates the observed plasma myo-inositol levels for fetuses, neonates, and young adult rats. With the exception of -2 days of age, significant differences ($p<0.025$) in plasma myo-inositol levels occurred between the two diets after birth (four days) through +14 days of age. At 21 days of age no significant differences were detected when comparing sex differneces between dietary groups or within dietary groups. After 21 days of age (weaning), significant differences in the plasma levels of myo-inositol were observed between males fed the deficient diet and males fed the supplemented diet ($p<0.025$); this was also the case for females fed the two diets ($p<0.025$). These differences, however, were not observed when comparing males and females fed the same diet. As previously

Figure 2. Free myo-Inositol Levels in the Plasma of A) Developing Rats and B) Lactating Dams. (A) Refer to figure 1 for the symbol legend. Each point represents the mean ± S.D. for four pools of fetuses (each pool from one dam), four pools of eight pups each for animals up to 21 days of age, four animals for 21 days of age, or three animals for ages greater than 21 days. (B) Each point represents the mean ± S.D. for four dams. ▲, supplemented diet; ●, deficient diet.

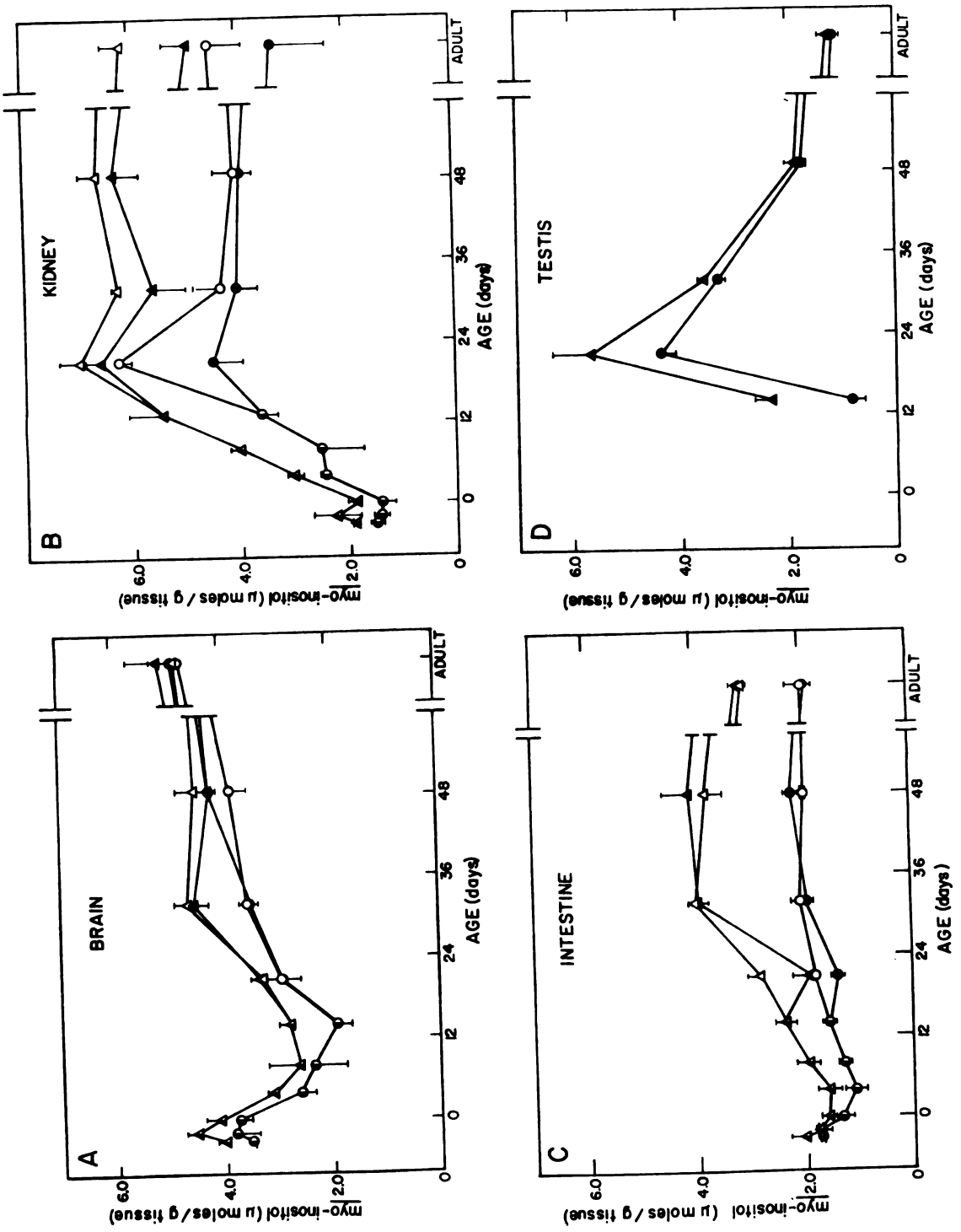

PLASMA
A
myo-inositol (mM)
0.6
0.4
0.2
0
0
12
24
36
48
ADULT
AGE (days)


PLASMA
B
myo-inositol (mM)
0.3
0.2
0.1
0
0
7
14
21
LACTATION (days)

reported (12), eight- to ten-fold differences in plasma myo-inositol levels were observed when comparing fetal to maternal values regardless of the myo-inositol content of the diet. An initial response to the milk of the supplemented dams (Figure 4A) was observed in the pups at eight days of age, but the decrease in plasma levels of myo-inositol postnatally continued at 14 days of age. Figure 2B indicates the levels observed of free myo-inositol in the plasma of pregnant and lactating dams fed the myo-inositol deficient and supplemented diets. Supplemented dams showed an elevation in the mean plasma myo-inositol content from three days prepartum (0.10 mM) to four days of lactation (0.26 mM). After seven days of lactation, plasma myo-inositol returned to prepartum levels though the difference in the means were not statistically significant between groups or with time. Deficient dams showed a similar pattern for plasma myo-inositol but without the subsequent rise from 0 to 4 days of lactation.

Brain and Kidney Free myo-Inositol. Differences in the developing rat brain free myo-inositol levels (Figure 3A) were noted throughout the period studied. Statistically significant differences between diets were observed at four ($p<0.05$), 14 ($p<0.02$), and 32 days of age ($p<0.05$). The physiological significance of the decrease in free myo-inositol levels after birth and the subsequent rise to levels greater than or equal to fetal brain levels remains obscure. Similarities between these studies and earlier work by Wells, et al. (18) and Allison and Stewart (19), were observed. In addition, the evidence indicates that the postnatal myo-inositol increase occurred more rapidly for the animals fed the supplemented diet than for the animals fed the deficient diet.

Figure 3. Free myo-Inositol Levels in Selected Tissues of Developing Rats Fed a myo-Inositol Supplemented or Deficient Diet. (A) Brain; (B) kidney; (C) small intestine; (D) testis. Supplemented rats ▲, males; △, females; ◭, a pool of males and females and deprived rats ●, males; ○, females; ◐, a pool of males and females. Each point represents the mean ± S.D. for four pools of fetuses (each pool from one dam), four pools of eight pups each for animals up to 21 days of age, four animals for 21 days of age and three animals for ages greater than 21 days.


A
BRAIN
B
KIDNEY
C
INTESTINE
D
TESTIS
myo-inositol (μ moles / g tissue)
AGE (days)
ADULT
0
12
24
36
48
2.0
4.0
6.0

The free myo-inositol content of kidney tissue is shown in Figure 3B. The postnatal elevation of free myo-inositol in kidney and a plateauing of the levels after 21 days of age were observed for both diets. Differences in kidney free myo-inositol levels at birth were not significant. Significant differences were observed for free myo-inositol levels at all ages examined prior to 21 days of age ($p<0.05$). There were significant differences ($p<0.02$) in free myo-inositol levels between males fed the supplemented diet and males fed the deficient diet; this was also true when comparing the females fed the different diets ($p<0.02$) with the exception of the 21 day old females.

Intestinal Free myo-Inositol Levels. The developing rat showed an approximate two-fold elevation in the intestinal free pool of myo-inositol regardless of the diet fed (Figure 3C). No significant differences in myo-inositol levels were noted prior to eight days of age; however, these intestinal samples were contaminated with intralumenal contents. Differences in myo-inositol levels were not observed between males and females fed the same diet with the exception of 21 day old animals when sex differences were significant ($p<0.02$) in both supplemented and deficient diets. At all ages after eight days, there were significant differences ($p<0.02$) in myo-inositol content between females fed the supplemented diet and females fed the deprived diet; this was also true for the males fed the different diets with the exception of the 21 day old males. The overall trend involving the increase in levels of myo-inositol in intestine reflected that observed in kidney; however, the plateau began somewhat later, at 32 days of age.

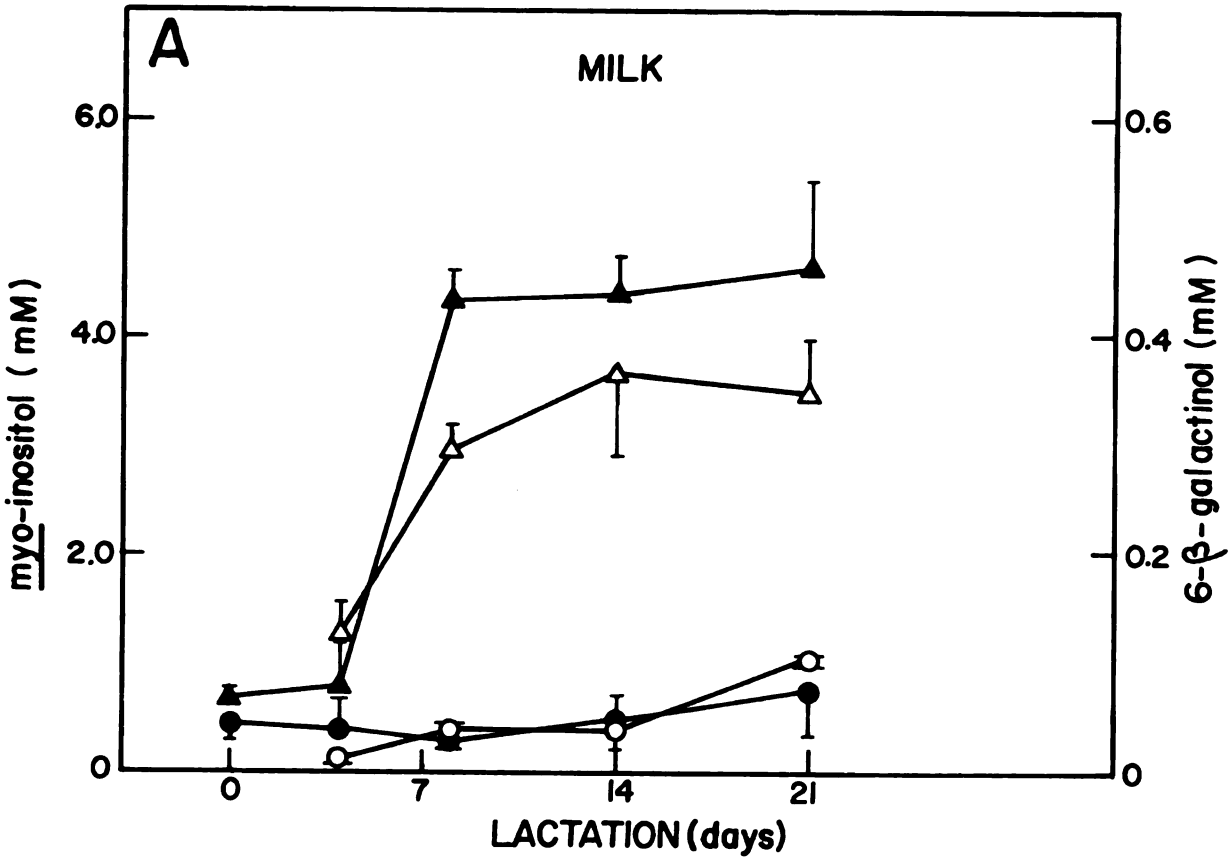
Fertility Studies. Because the free myo-inositol level of rat gonads is normally high with respect to other tissues, we examined

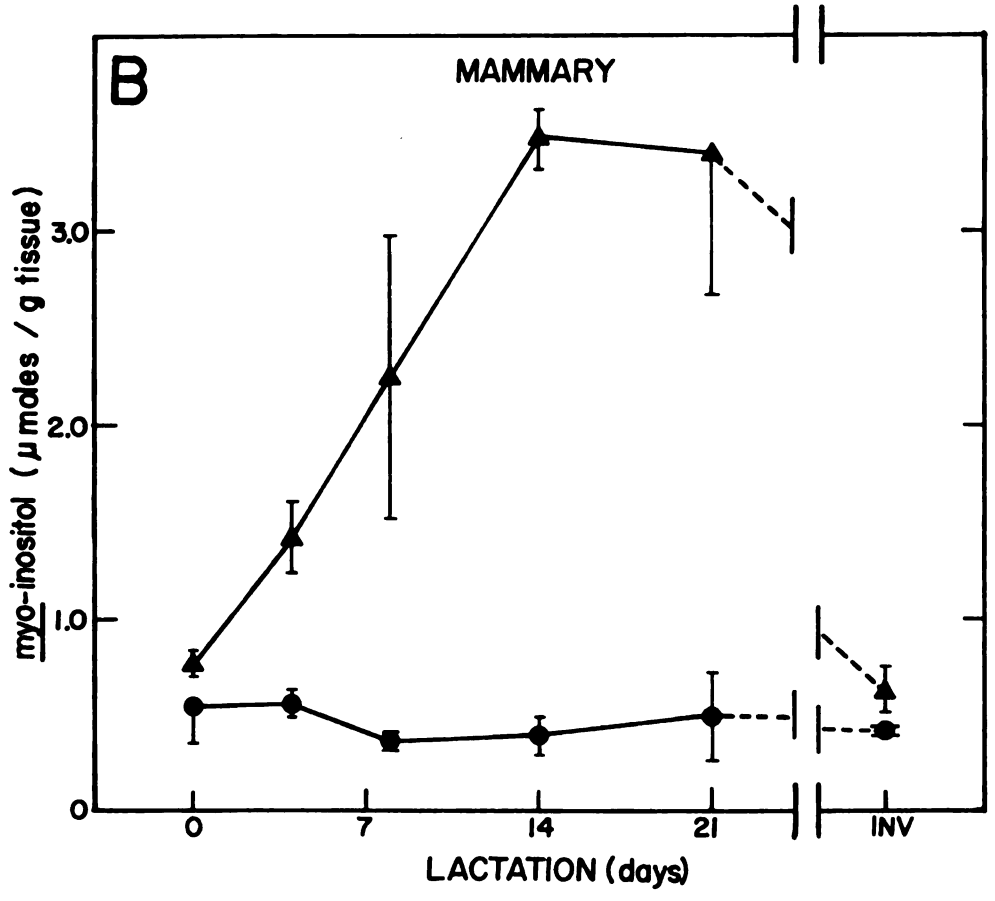
the effects of the deficient diet on those levels and on the sperm count. Figure 3D shows the free myo-inositol levels for the developing rat testis. The diet significantly ($p<0.05$) affected levels of myo-inositol in this tissue prior to 32 days of age. Prior to 49 days of age sperm counts were not measureable by the visual counting technique used. Supplemented 49 day old males gave counts of $1.30 \pm 0.11 \times 10^8$ sperm per testis or $0.94 \pm 0.04 \times 10^8$ sperm per gram tissue, whereas corresponding myo-inositol deprived males were $1.21 \pm 0.17 \times 10^8$ sperm per testis or $0.93 \pm 0.11 \times 10^8$ sperm/g tissue. Supplemented adult males revealed higher values of $2.21 \pm 0.08 \times 10^8$ sperm per testis or $1.248 \pm 0.130 \times 10^8$ sperm/g tissue. Sperm counts for deprived adults were $2.13 \pm 0.12 \times 10^8$ sperm per testis or $1.201 \pm 0.031 \times 10^8$ sperm/g tissue ($n=3$ for all of the above observations). These observations neither support nor discount potential effects of myo-inositol on the fertility of rats since the presence of sperm was observed only after early differences in myo-inositol levels disappeared.

Fertility tests were conducted to determine whether possible indirect effects of early testicular myo-inositol differences affected fertility. Of the 12 males and 12 females from each of the two diets examined, all were shown to be fertile with no significant differences in litter sizes.

Effects on Milk myo-Inositol. Figure 4A shows the content of free myo-inositol in rat milk as affected by the diets. For the animals receiving the deficient diet, low levels (0.30 to 0.75 mM) were observed throughout the lactational period. A slow rise in the mean free myo-inositol content of the milk from 8 to 21 days of lactation was

Figure 4. Effects of Dietary myo-Inositol on A) Milk and B) Mammary Gland Free myo-Inositol Levels, and A) Milk 6-β-Galactinol Levels. myo-Inositol levels in the milk or mammary gland are represented as follows: ▲, supplemented dams; ●, deprived dams. 6-β-Galactinol levels in the milk are represented by: △, supplemented dams; ○, deprived dams. Each point represents the mean ± S.D. for four dams. INV refers to involution of 28 days.


A
MILK
6.0
4.0
2.0
0
0.6
0.4
0.2
0
myo-inositol (mM)
6-β-galactinol (mM)
0
7
14
21
LACTATION (days)


B
MAMMARY
3.0
2.0
1.0
0
myo-inositol (μmoles / g tissue)
0
7
14
21
INV
LACTATION (days)

observed, however; this may be correlated with an increased rate of biosynthesis as reported in previous work (12). The milk from those animals receiving the supplemented diet showed a 5.5-fold increase in myo-inositol levels at day eight ($p<0.001$) when compared with day four of lactation. When compared with deficient diet fed animals, the milk myo-inositol level from myo-inositol supplemented dams remained relatively constant (4.5 mM) to day 21 ($p<0.001$, supplemented versus deprived dams). Although both supplemented and deprived mothers received their respective diets continuously, no significant difference was noted in the myo-inositol content of their milk between birth and the fourth day of lactation.

The mammary tissue content of free myo-inositol (Figure 4B) followed a pattern closely related to that of the milk and similar to previous observations (12). However, the plateau observed in the supplemented animals' milk (8 - 21 days of lactation) was not observed in the gland until somewhat later (14 - 21 days of lactation). In the supplemented animals' mammary tissue, however, no delay in the increase of free myo-inositol levels from 0 to 4 days of lactation occurred. Involuted mammary tissue showed a small but significantly ($p<0.05$) higher content of myo-inositol for the supplemented animals (0.63 umoles/g tissue) when compared with the deficient animals (0.43 umoles/g tissue).

Effects on 6-β-Galactinol Content of Milk. Previous work (20) has shown a relationship between the milk free myo-inositol content and the levels of 6-β-galactinol in the milk. Figure 4A shows that the pattern of 6-β-galactinol content follows the pattern for myo-inositol content very closely in both the milk from control or myo-inositol

TABLE 1

EFFECT OF DIETARY myo-INOSITOL ON THE FREE myo-INOSITOL AND 6-β-GALACTINOL CONTENT OF RAT MILK AT 14 DAYS OF LACTATION

Dietary Level of myo-Inositol (%)	myo-Inositol (mM)	6-β-Galactinol (uM)
0	0.492 ± 0.253 (4)	36.4 ± 19.5 (4)
0.01	0.617 ± 0.012	40.9 ± 2.6
0.05	0.964 ± 0.041	63.1 ± 9.3
0.25	4.014 ± 0.244	292.0 ± 16.8
0.50	4.422 ± 0.366 (4)	365.6 ± 76.5 (4)

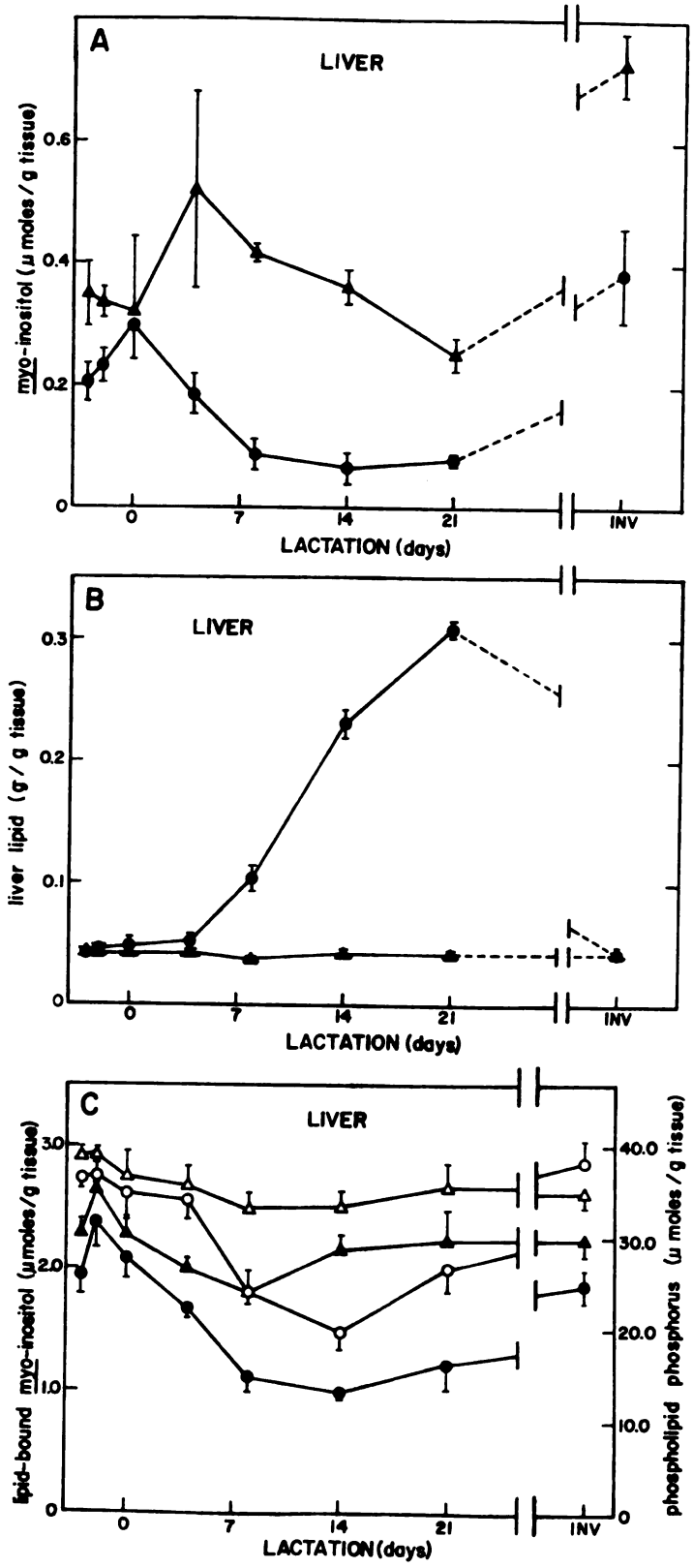
Values are the mean ± S.D. for 3 dams unless otherwise indicated (N). Females were fed a synthetic diet with the appropriate level of free myo-inositol and myo-inositol and 6-O-β-D-Galactopyranosyl-inositol were determined by gas-liquid chromatography as described in the Materials and Methods section.

deprived dams. 6-β-Galactinol levels in the milk of the myo-inositol supplemented dams was 3.5 - 9 times greater than that in the milk of deprived dams (statistically significant at all points, $p < 0.05$).

Effects on 6-β-galactinol were also shown by varying the myo-inositol content of the diet through a range of values (see Table 1). 6-β-Galactinol was shown to increase as the myo-inositol in the milk increased and maximized when the higher dietary levels of myo-inositol were fed.

Effects of Lactation on Dams. The free myo-inositol content of maternal liver from the third day prior to birth through 21 days of lactation and four weeks after weaning is shown in Figure 5A. Livers of supplemented dams showed no significant change in the level of free myo-inositol during gestation and at birth (approximately 0.33 umoles/g fresh weight tissue). At four days of lactation, levels increased to over 0.50 umoles/g tissue concomittant with plasma myo-inositol increases during the same period. After four days of lactation, mean free myo-inositol levels steadily decreased to below prelactational levels by 21 days of lactation (0.25 umoles/g tissue). However, when compared with animals at 0 days of lactation, these changes during the course of lactation were not significant. Mean myo-inositol levels in the liver of the dams four weeks after weaning were significantly ($p < 0.05$) higher than at any other time examined for both the myo-inositol supplemented and deprived animals with the single exception of supplemented animals at four days of lactation. The livers of deprived dams showed a rising trend (not statistically significant) in mean free myo-inositol content during gestation and at birth. After birth and the initiation of lactation, however, liver mean free myo-inositol

Figure 5. Effects of Dietary myo-Inositol on A) Free and C) Lipid-Bound myo-Inositol, and B) Total Lipid, and C) Phospholipid Phosphorus Content in the Liver of Lactating Dams. Symbols represent as follows: lipid-bound myo-inositol levels; ▲, supplemented diet fed animals and ●, deficient diet fed animals; phospholipid phosphorus levels; △, supplemented diet fed animals and ○, deficient diet fed animals. Each point represents the mean ± S.D. for four dams. In (C), values are expressed in umoles/ g lipid-free tissue. INV refers to involution of 28 days.


A
LIVER
myo-inositol (μ moles / g tissue)
0.6
0.4
0.2
0
0
7
14
21
INV
LACTATION (days)
B
LIVER
liver lipid (g / g tissue)
0.3
0.2
0.1
0
0
7
14
21
INV
LACTATION (days)
C
LIVER
lipid-bound myo-inositol (μmoles/g tissue)
3.0
2.0
1.0
0
phospholipid phosphorus (μ moles / g tissue)
40.0
30.0
20.0
10.0
0
0
7
14
21
INV
LACTATION (days)

levels significantly ($p < 0.005$, compared with day 0 of lactation) decreased to a level of 0.07 umoles/g tissue by eight days of lactation where it remained until weaning (21 days of lactation). Recovery of free myo-inositol levels to pre-lactational levels (0.38 umole/g tissue) was also observed for the myo-inositol deprived dams.

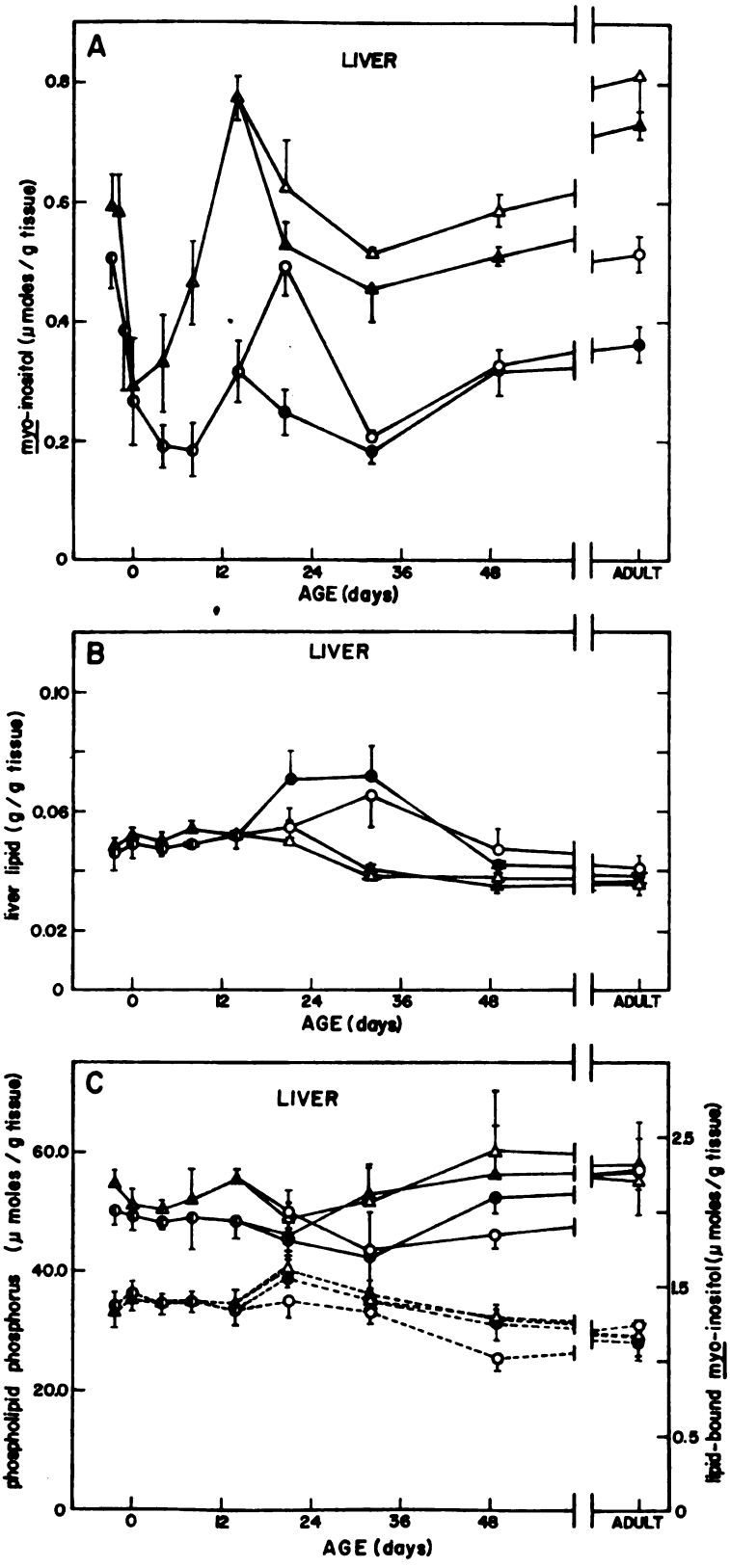
Development of Fatty Liver in Lactating Dams. The change in the percent lipid content of the livers of lactating dams was the most dramatic change observed during this study (Figure 5B). myo-Inositol supplemented dams showed no change in liver lipid content (0.045 g lipid/g liver) during gestation, lactation, or after involution. Deprived dams exhibited normal levels of liver lipid during gestation through the fourth day of lactation, after which a nearly linear rise in the lipid content of the liver was observed until 21 days of lactation (0.31 g lipid/g fresh weight of liver). After involution, the lipid content of the livers from myo-inositol deprived animals returned to normal levels. Analyses were undertaken to examine the effects of the changes in the free myo-inositol and lipid content on the phospholipid (Figure 5C) and the phosphatidylinositol levels of the livers of lactating dams. The livers of supplemented dams showed only insignificant variations from a mean value of 35 umoles of phospholipid phosphorus per gram of lipid-free liver; livers from deprived dams were equivalent to those from supplemented animals for the first four days of lactation. A steady decline in the content of phospholipid phosphorus after four days of lactation to a minimum (20 umoles per gram lipid-free liver, $p < 0.005$, when comparing livers from supplemented and deprived rats) at 14 days of lactation in livers of deprived dams was followed by a partial recovery to 27 umoles per gram of lipid-free

tissue at 21 days of lactation ($p<0.02$, day 14 versus day 21). After weaning, livers from dams showed no significant change from the mean value of 35 umoles/g lipid-free liver for the supplemented dams and full recovery of the myo-inositol deprived dams occurred by 28 days after weaning (38 umoles per gram of lipid-free liver).

The mean level of lipid-bound inositol in the livers of myo-inositol supplemented dams tended to decline (not statistically significant) from two days prior to birth to a minimum of 1.8 umoles per gram of lipid-free liver at 14 days of lactation which persisted until after involution. A parallel decrease of phosphatidylinositol in livers of myo-inositol deprived rats occurred during lactation (1.0 umoles per gram of lipid-free liver at 14 days of lactation ($p<0.001$) when comparing supplemented and deprived animals). A partial recovery to 1.25 umoles per gram of lipid-free tissue occurred by 21 days of lactation, ($p<0.01$, supplemented versus deprived animals) and after weaning, the tissue showed levels of 1.85 umoles of phosphatidyl-inositol per gram of lipid-free tissue ($p<0.05$, supplemented versus deprived animals). These observations suggest that myo-inositol deprivation may delay the return of lipid-bound myo-inositol to normal levels.

Developing Rat Liver Free myo-Inositol. Fetal liver levels of free myo-inositol (Figure 6A) tended to parallel the levels observed in the fetal rat plasma (Figure 2A). Supplemented animals showed a precipitous drop in liver myo-inositol levels from fetal ages (-2 and -3 days) to a minimum of 0.3 umole per gram of fresh tissue at birth. Following birth, a sharp rise (to a maximum of 0.8 umole/g tissue) in the liver free pool was observed closely following the dietary intake

Figure 6. Effects of Dietary myo-Inositol on the A) Free and C) Lipid-Bound myo-Inositol, B) Total Lipid, and C) Phospholipid Phosphorus Levels in the Liver of the Developing Rat. Supplemented animals ▲, males; △, females; ◭, a pool of males and females and deprived animals ●, males; ○, females; ◐, a pool of males and females. Each point represents the mean ± S.D. for four pools of fetuses (each pool from one dam), four pools of eight pups each for animals up to 21 days of age, four animals for 21 days of age and three animals for ages greater than 21 days. In C), solid lines represent lipid-bound myo-inositol levels and dashed lines represent phospholipid phosphorus levels expressed as umoles/ g lipid-free tissue.


A
LIVER
myo-inositol (μ moles / g tissue)
0.8
0.6
0.4
0.2
0
0
12
24
36
48
ADULT
AGE (days)
B
LIVER
liver lipid (g / g tissue)
0.10
0.06
0.02
0
0
12
24
36
48
ADULT
AGE (days)
C
LIVER
phospholipid phosphorus (μ moles / g tissue)
60.0
40.0
20.0
0
lipid-bound myo-inositol (μ moles / g tissue)
2.5
1.5
0.5
0
0
12
24
36
48
ADULT
AGE (days)

until 14 days of age. From 14 to 32 days of age, there was decrease in the liver free pool of myo-inositol followed by a subsequent rise to adult levels approximately equal to levels observed at 14 days of age. Deprived animals showed a lowered but essentially parallel pattern. The neonatal rise in myo-inositol levels observed in supplemented pups did not occur in deficient pups until after eight days of age, and the levels were significantly ($p < 0.02$) lower than corresponding controls for both sexes at all ages after 14 days with the single exception of 21 days supplemented and deprived females.

Developing Rat Liver Lipid, Lipid-Bound myo-Inositol and Phospholipid Phosphorus Content. Figure 6B illustrates liver lipid contents in developing rat pups fed the deficient and supplemented diets. Differences in liver lipid content between groups were not significant. Males fed the myo-inositol deficient diet showed a tendency for an increase in liver lipid from 5.0% to 7.0% on a fresh weight basis compared with supplemented males at 21 days of age. At 32 days of age, an increase from 5.0% at 21 days to 7.0% at 32 days for deprived males and from 5.0% at 21 days to 6.5% at 32 days for deprived females was observed. This was the only instance that significant differences ($p < 0.05$) between dietary groups were noted for either males or females.

No significant differences were observed in the phospholipid phosphorus or lipid-bound myo-inositol content in liver due to diet, sex or age of the animal, with the exception of liver phospholipid phosphorus of supplemented and deprived females at 49 days of age ($p < 0.05$) (Figure 6C).

Fatty Acid Distribution in Total Liver Lipids. Table 1 shows the

TABLE 2

FATTY ACID DISTRIBUTION OF DIETARY LIPID AND TOTAL LIPIDS IN LIVER OF myo-INOSITOL DEFICIENT AND SUPPLEMENTED LACTATING RATS

Sample	Fatty Acids (% Total Lipids)									
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	Other	Sat./Unsat.
Diet (soybean oil)	---	11.9 ± 1.4	0.6 ± 0.1	4.0 ± 0.1	24.4 ± 1.3	52.2 ± 2.0	6.9 ± 0.4	---	---	0.19
myo-Inositol Supplemented	0.8 ± 0.0	28.5 ± 1.7	4.7 ± 0.5	16.5 ± 1.6	31.3 ± 3.2	7.6 ± 0.7	0.3 ± 0.1	10.3 ± 2.0	0.1 ± 0.1	0.85
myo-Inositol	1.4 ± 0.3	30.0 ± 2.8	7.9 ± 0.2	3.7 ± 0.1	44.9 ± 3.3	7.2 ± 1.7	0.7 ± 0.4	1.1 ± 0.2	0.1 ± 0.1	0.62

Values are expressed as the mean ± S.D. of samples from 4 rats or 4 diet samples. Liver samples were taken at 21 days of lactation. The fatty acid methyl esters are identified according to carbon atom number: number of double bonds.

results of gas-liquid chromatographic analysis of the total liver lipid of lactating rats fed a deficient or supplemented diet for five weeks (21 days of lactation) and the fatty acid content of the vegetable oil used in the diet. The lipid from the livers of myo-inositol deprived dams was significantly increased in 14:0 ($p<0.02$), 16:1 ($p<0.005$) and 18:1 ($p<0.01$) fatty acids and decreased in 18:0 ($p<0.001$) and 20:4 ($p<0.005$) fatty acids when compared with animals fed the supplemented diet. No significant changes occurred in relative amounts of 16:0, 18:2 and 18:3 fatty acids.

DISCUSSION

Most nutritional work regarding myo-inositol has centered around its role as an essential nutrient in microorganisms (20 - 22) and mammals (2, 23, 24). Original studies by Woolley (25, 26) in the mouse demonstrated inadequate growth, alopecia, and eventual death. Later, Martin (27) revealed discrepancies in this work, and Woolley (28, 29) suggested a possible relationship between myo-inositol and pantothenate to explain the contradictory observations. Early studies on the dietary requirement of the rat for myo-inositol also gave contradictory results (30 - 35). Exogenous sources, including impure diets (35, 36) and intestinal flora biosynthesis (37) contribute to the difficulty of interpreting these studies. In addition, endogenous sources, which include in vivo synthesis (36) and possible tissue mobilization of myo-inositol, present problems in the study of its dietary role.

The purpose of the present study was to examine the effects of a myo-inositol deficient diet on the development of the rat. Our aim was

to eliminate as many variables as possible in order to more properly interpret the role of myo-inositol in these biological functions. A relationship between myo-inositol and other vitamins has been suggested (38, 39). Therefore, the diet used in this study was designed to contain adequate amounts of all known vitamins, choline, and protein, as well as phthalylsulfathiazole as an antibacterial agent. Work with fat-free diets and the influence of various fats (9, 40, 41) on the lipotropic effects of myo-inositol prompted us to use a dietary fat source which contained adequate amounts of essential fatty acids.

It was also important to demonstrate that a reduction of tissue myo-inositol levels and phosphatidylinositol occurred in the animals fed a myo-inositol deficient diet. Generally, the deprived animals showed depressed levels of free myo-inositol in the tissues and fluids examined. The development of the rat was followed from fetus (-3 days) through adulthood (120 days), and differences in the tissue levels of the supplemented versus the deprived pups, though varying in magnitude, were maintained for the duration of the experimental period. Rat pup plasma myo-inositol (Figure 2) followed the previously reported developmental pattern in the rat (10). Brain free myo-inositol levels (Figure 3A) mimicked those previously reported (18, 19). However, at 14 days and 32 days of age, myo-inositol levels in the deprived pups were significantly lower than those in normal pups. In pup kidney as well as intestine, (Figures 3B, 3C), significant differences were observed during development; differences between males and females within a particular diet occurred only at 21 days of age. In pup liver (Figure 6A), developmental profiles reflected the dietary intake of myo-inositol until weaning (21 days of age) when the conversion was made to a purified diet containing a sulfa drug. The alteration of

the developing intestinal flora (ingestion of a sulfa drug) may be the major cause for the subsequent myo-inositol decrease in the liver of pups fed the deficient diet.

Effects of myo-inositol deprivation on fertility were observed by Ershoff and McWilliams (42), but only after addition of a sulfa drug. They observed no effects on growth or reproduction by the addition of myo-inositol to the diet. Hamilton and Hogan (43), however, observed severe problems during parturition, including stillborn young or the birth of formless masses during the feeding of myo-inositol deficient diets to hamsters. The high levels of myo-inositol known to be present in rat testes prompted us to examine possible dietary effects on the fertility of rats. Figure 3D and sperm count data present evidence that in the male rat, infertility does not occur during the feeding of a myo-inositol deficient diet. This is not surprising because Middleton and Setchell (44) have reported the significance of in vivo synthesis of myo-inositol in ram testes as opposed to accumulation from the plasma. In addition, high levels of D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) reported in rat testes (45) support the observed ineffectiveness of the myo-inositol deficient diet to alter the levels of testicular myo-inositol in the sexually mature rat.

Our data show a strong correlation between the free myo-inositol content of the diet and free myo-inositol level in the milk of the dam (Figure 4A). myo-Inositol deprived dams showed the lowest levels thus far reported for rat milk during lactation (12, 20). The effect of dietary myo-inositol on the myo-inositol level in the milk was evident as early as the fourth day of lactation in the myo-inositol

supplemented dams; the maximum level (4.5 mM) was reached by the eighth day of lactation. The mechanism of transport of myo-inositol from plasma to mammary tissue remains undetermined although myo-inositol transport systems were identified in kidney by Hauser, et al. (46). Mobilization of free myo-inositol to the mammary gland and milk from other tissues and the plasma may contribute to the content of myo-inositol in the milk. The appearance of myo-inositol in the milk and mammary gland occurred simultaneously with the decrease of the mean free pool of myo-inositol in the livers of both myo-inositol deprived and supplemented dams. However, the decrease in mean values was not statistically significant with time. Decreased bound myo-inositol and reduced phospholipid phosphorus in the deprived livers were also observed during the lactation period. After weaning, the liver free myo-inositol content returned to normal in both supplemented and deprived dams as did the fatty liver in the deprived dams; this supplies further evidence of the depletion effect on the liver during lactation. It was of interest to determine the effect of dietary myo-inositol concentration and 6-β-galactinol in rat milk. The data (Figure 4A and Table 1) supported previous enzymatic studies (20, 47) showing a relationship between mammary myo-inositol concentration and 6-β-galactinol production in the presence of β-galactosidase (EC 3.2.1.23) and excess lactose.

myo-Inositol deprivation manifested itself in the production of fatty liver in the lactating dams. Previous investigators (9) have reported the development of fatty liver in the rat using a similar dietary regimen. However, in those studies, to produce a fatty liver in young rat pups, it was necessary for the dietary fat to be saturated. Utilizing a diet containing adequate amounts of essential fatty acids,

protein, choline, and other vitamins, we have discovered a requirement for dietary myo-inositol for the prevention of lactation induced fatty liver. Corresponding myo-inositol supplemented controls maintained normal liver lipid levels of 4.5% throughout lactation (Figure 5B). Lipid deposition in deprived livers was accompanied by depressed phospholipid phosphorus (57% of control livers) and depressed phosphatidylinositol (50% of control levels) levels (Figure 5C). The increase in lipid was predominantly in the triglyceride fraction[8]. Complete recovery of phospholipid phosphorus was observed after involution, but recovery of lipid-bound liver myo-inositol was incomplete.

Hayashi, et al. (9) suggest that the reason for liver lipid buildup in the absence of myo-inositol is the mobilization of fat to the liver beyond the liver's normal ability to process and export lipids. Our preliminary experiments do not distinguish between this mechanism and others including the possibility of a reduction in the liver's ability to process lipoproteins for export to the plasma and mammary gland in addition to an increased mobilization of free fatty acids from adipose tissue (48). Our evidence does suggest, however, that there is a relationship between lactation and the supply of plasma lipoproteins that is abnormal in dietary myo-inositol deprivation. Other studies have shown similar effects of demands on the liver during myo-inositol deprivation in laying hens (49). Therefore, "physiological stress" may require amounts of myo-inositol beyond that necessary for normal liver function. In our studies, the effect of lactation on fatty liver may also be unusually vulnerable to other dietary deficiencies or toxic agents.

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[8] L.E. Burton and W.W. Wells, unpublished results (1976)

Since the fatty liver condition developed only during lactation in myo-inositol deprived dams fed diets containing adequate amounts of essential fatty acids, protein, other vitamins, and choline, we conclude that there is a close dependency on the liver for delivery of lipoproteins during lactation in the rat. It is known that over 50% of milk triglyceride is derived from plasma lipoproteins (50), most of which, in turn, is derived from the liver. The elevation in liver triglyceride resulting from dietary myo-inositol deprivation may be caused by increased free fatty acid mobilization from fat depots to the liver in response to lactation, coupled to the inability of the liver to produce normal quantity or quality of lipoproteins due to insufficient phosphatidylinositol. The liver levels of phosphatidylinositol observed during lactation-induced fatty liver were approximately 1.0 umole/g fresh tissue (Figure 5C), or only one-half that of control animals. Increased lipoprotein synthesis and export by the liver during lactation must require more phosphatidylinositol than the rat can provide from endogenous sources. Thus, myo-inositol has been found to be a dietary factor that is required to prevent lactation-induced fatty liver in the rat. The precise mechanism leading to this fatty liver condition remains to be elucidated.

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CHAPTER IV

CHARACTERIZATION OF THE LIPID IN myo-INOSITOL DEFICIENT LACTATION-INDUCED FATTY LIVER AND PLASMA IN RATS

ABSTRACT

Characteristics of the lipid deposited during myo-inositol deficient lactation-induced fatty liver development have been studied. After four days of lactation, cholesterol esters (CE) and triglycerides (TG) were significantly elevated ($p<0.05$) in the livers of myo-inositol deprived dams when compared with animals fed myo-inositol supplemented diet whereas liver phospholipids (PL) of deprived dams were significantly ($p<0.05$) depressed during the same period. Electronmicroscopy revealed the presence of intracellular lipid droplets in the livers of deprived dams which correlated well with the increase in liver fat content.

Plasma lipoprotein-associated lipids (C, CE, TG, PL) were generally depressed for deprived dams during lactation suggesting a block in lipoprotein secretion. In addition, elevations of the same lipids after the onset of involution emphasized the role lactation and associated hormonal changes may play in the maintenance of the fatty liver. Elevation of plasma free fatty acids (FFA) in deprived dams above that of the supplemented dams was also observed during the course of lactation. The role of each of these contributors to the development of myo-inositol deficient lactation-induced fatty liver is discussed.

No changes in lipid content as a result of myo-inositol deprivation or lactation were observed in kidney and intestinal tissues from 21 day lactating dams fed the deficient and supplemented diets.

INTRODUCTION

The previous chapter (Chapter III) has reported a myo-inositol deficient lactation-induced fatty liver produced in rats which was alleviated by myo-inositol supplementation or termination of lactation. Hegsted et al. (1, 2) have reported an intestinal lipodystrophy in the female gerbil which is dependent upon the feeding of a myo-inositol deficient diet and the saturation of the fat constituent in the diet. In addition, Hayashi, et al. (3) have reported a myo-inositol deficient fatty liver in weanling male rats which was also dependent on dietary saturated fats. These types of myo-inositol deficient fat deposition are different from that of the lactation-induced fatty liver in that it was not shown to be saturated fat dependent and was reversible at the termination of lactation despite the continued feeding of a myo-inositol deficient diet.

This chapter describes the characterization of the fat deposited in the myo-inositol deprived lactation-induced fatty liver (4) and the plasma lipids in the lactating rat. The study has followed the development of the fatty liver during lactation and at selected time points during involution. The resulting data will be discussed with regard to the possible mechanism(s) of this type of fatty liver.

MATERIALS AND METHODS

Materials. Dietary materials were from sources indicated in Chapter III. Gas chromatographic materials included those listed in the previous chapter as well as: 3% XE-60 (w/w) on Gaschrom Q (100-120 mesh) from Supelco, Inc.; 5-α-cholestane from Schwartz/Mann; and cholesterol from Sigma Chemical Company.

Animal Treatments and Diets. The composition of the diet has been detailed elsewhere (see Chapter III). Liver and plasma samples were from the same animals examined in the previous chapter.

Total Lipid Extractation and Quantitation. Total lipid was extracted from liver or plasma samples by the method of Folch et al. (5) and liver lipid contents were determined gravimetrically. All organic solvents used in the extractions were freshly redistilled.

Free and Esterified Cholesterol. Free and esterified cholesterol in liver and plasma were determined by the gas chromatographic method of Schmit et al. (6) utilizing a 1.8m by 3mm column of 3% XE-60 (w/w) on Gaschrom Q (100-120 mesh) at 235° C. Determinations were made from the total lipid extract rather than from whole plasma or tissue.

Triglycerides and Glucose Levels. Triglycerides were determined from total lipid extracts and glucose from whole plasma using the Gilford Systems 3500 Computer Directed Analyzer and the Worthington/Gilford Automated Triglyceride Analysis Kit or the Worthington/Gilford Glucose Kit.

Phospholipid Quantitation. Phospholipid phosphorus in the extracted lipids of liver or plasma was measured by the method of Ames (7).

Phosphatidylinositol Levels. Phosphatidylinositol in plasma and liver was determined from the extracted lipids by the method of Wells et al. (8).

Free Fatty Acids. Free fatty acids in plasma were determined by the Antonis modification (9) of the Duncombe method (10). Twice recrystallized stearic acid was used as the standard fatty acid.

Summation of Total Lipids. Total lipids in the plasma of the lactating dams were determined by summation of the separate analyses

TABLE 1

Relationship of Liver and Body Weight During Lactation and Involution for Dams Fed a myo-Inositol Supplemented or Deficient Diet

Animals	Liver as a % Body Weight[a]						
	Days of Lactation				Days of Involution		
	4	8	14	21	4	7	28
Supplemented Dams	4.3 ± 0.1	4.4 ± 0.3	5.2 ± 0.1^{b}	5.5 ± 0.3^{b}	-----	-----	4.3 ± 0.2
Deprived Dams	4.6 ± 0.3	5.2 ± 0.2$^{c}_{C}$	7.0 ± 1.1$^{b}_{D}$	8.8 ± 1.5$^{b}_{B}$	7.6 ± 0.3^{c} (3)	5.7 (1)	4.3 ± 0.2

[a]Each value is the mean ± S.D. for 4 animals except where indicated (N). Statistical significance is indicated (using the Student's t test) as follows: b. $p<0.02$, c. $p<0.025$, d. $p<0.05$. Lower case letters represent comparisons within a diet based on 28 day involution values. Upper case letters represent comparisons between diets.

for the major lipid classes. Lipid levels were calculated as follows: triglycerides were converted to mg utilizing an average molecular weight of 885; phospholipids were determined on the basis of umoles of phospholipid phosphorus and converted from umoles to mg using a factor of 25; and values for free cholesterol were used as determined while esterified cholesterol determined as mg of cholesterol was converted assuming an average molecular weight of 284 (C_{18}) for the fatty acid ester.

Electron Microscopy. Thin slices of the liver were fixed for 2½ hours in 5% (v/v) glutaraldehyde in 0.1 M potassium phosphate, pH 7.2, postfixed 1½ hours in buffered 1% OsO_4 and dehydrated in graded aqueous ethanol prior to embedding in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips EM 300 microscope.

RESULTS

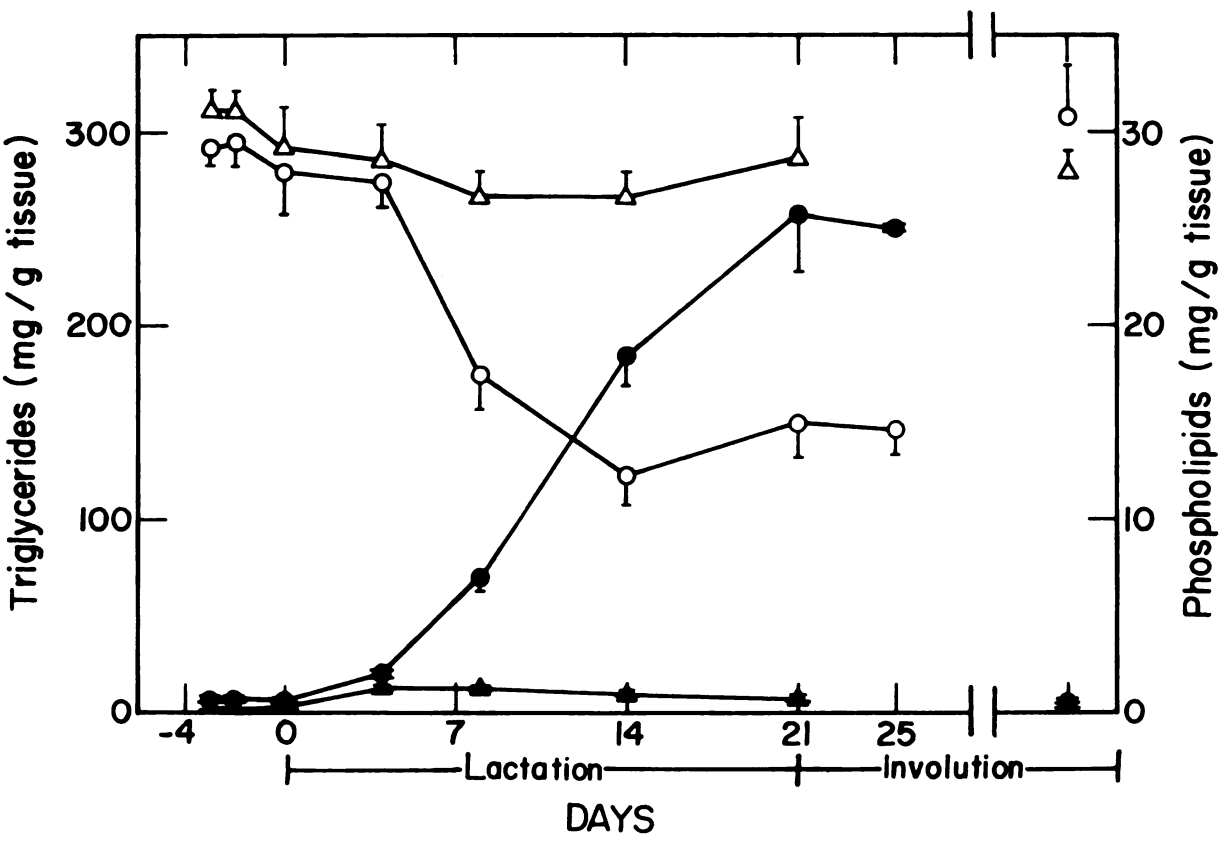
Effects of Dietary myo-Inositol on Liver Weight as a Percent of the Total Body Weight. Table 1 shows the relationship of liver and body weight during lactation and involution for dams fed a myo-inositol supplemented or deficient diet. Increases in liver weight during lactation have been previously described in rats (11). Supplemented dams showed a significant increase in liver weight as a function of total body weight at 14 ($p<0.02$) and 21 ($p<0.02$) days of lactation, returning to early lactation levels by 28 days post-weaning. Deprived dams also showed significant (8, $p<0.025$; 14, $p<0.02$; 21, $p<0.02$) increase in liver weight paralleling those observed for supplemented dams, however, the increase was from two-(at 8 to 14 days of lactation)

to six-fold (at 4 to 8 and 14 to 21 days of lactation) of that observed for the supplemented dams during the same period. Appendix Figure 1 reflects the apparent increase in the mass of the liver of the deprived dam when compared with a supplemented animal of similar body weight. The "yellowed" appearance of the deprived liver is typical of other types of fatty liver. Preliminary results have indicated no significant difference in the liver protein content when comparing the two groups at the same period in lactation (Burton, L.E. and Wells, W.W., unpublished, 1975).

Lipid Constituents of the Fatty Liver. Characteristics of the lipid deposited in the livers of lactating dams fed a myo-inositol deficient diet are presented in Figures 1 and 2. Four major classes of lipids were examined in the neutral total lipid extract. The greatest percentage (97% at 21 days of lactation) of the lipid deposited in the liver of the deprived dam was triglyceride (Figure 1). Significant differences in liver triglyceride content between supplemented and deprived dams began to occur after four days of lactation (8 days, $p<0.005$; 14 and 31 days, $p<0.001$) concommittant with the total lipid deposition previously observed (see Chapter III, Figure 5B). Phospholipids exhibited a significant decrease in the liver of the deprived dam when compared with the supplemented dam at 8 ($p<0.01$), 14 ($p<0.005$) and 21 ($p<0.005$) days of lactation (Figure 1). Recovery of both tryglyceride and phospholipid to control levels were observed after involution, although complete recovery occurred sometime after four days post-weaning. Previous work (Chapter III) showed significant depressions in liver phosphatidylinositol for deprived versus supplemented dams at 8 (p 0.005). 14 ($p<0.001$) and 21 ($p<0.01$) days of lactation coinciding with the depression in phospholipids but beginning earlier at four days of

Figure 1. Triglyceride and Phospholipid Content of Liver Lipids in Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents a mean ± S.D. for 4 animals except 25 days after birth where n=3. For triglyceride levels: ▲, supplemented dams; ●, deprived dams. For phospholipid levels: △, supplemented dams; ○, deprived dams. Values were not determined for supplemented animals 25 days after birth.

Figure 2. Free and Esterified Cholesterol Content of Liver Lipids in Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point is the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as mg of free (△, supplemented dams; ○, deprived dams) or esterified (▲, supplemented dams; ●, deprived dams) cholesterol / g of tissue. Values were not determined for supplemented animals 25 days after birth.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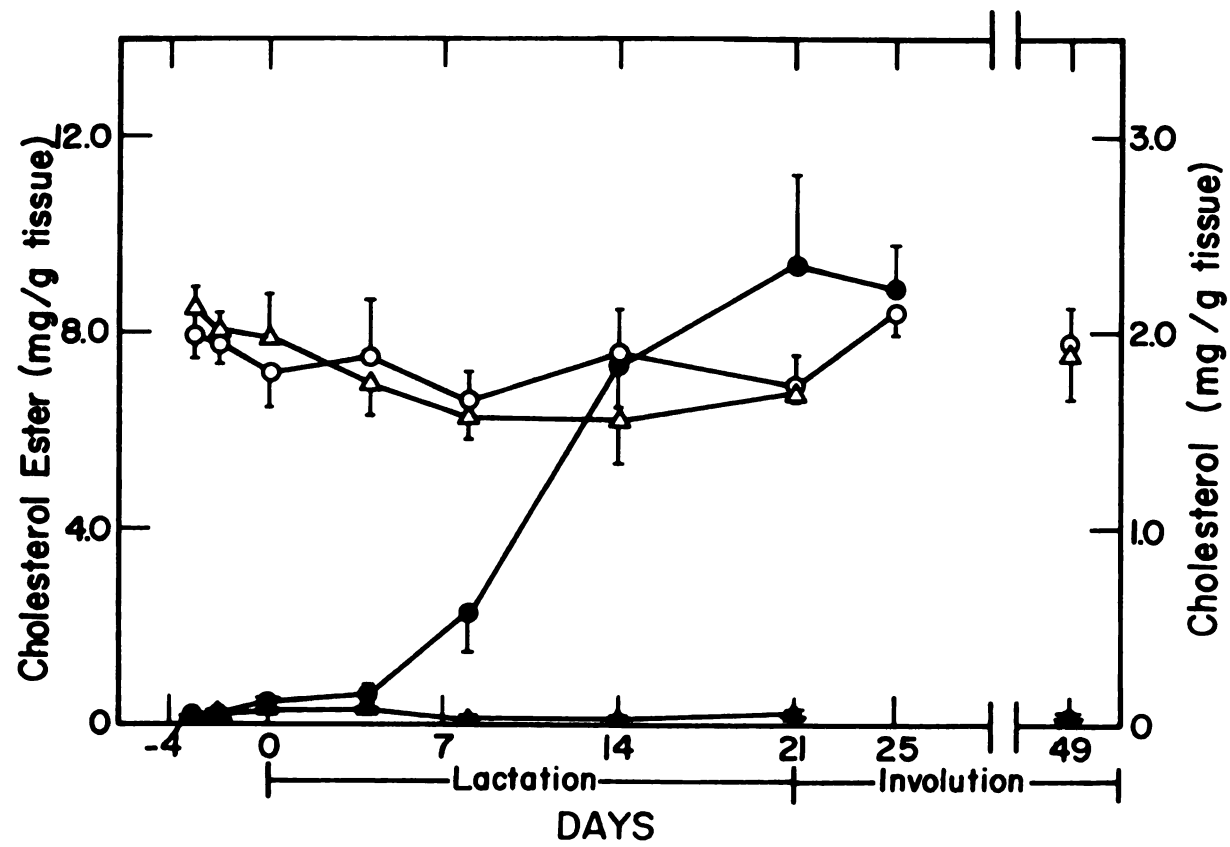
300
200
100
0
Phospholipids (mg/g tissue)
30
20
10
0
-4
0
7
14
21
25
Lactation
Involution
DAYS


Cholesterol Ester (mg/g tissue)
12.0
8.0
4.0
0
Cholesterol (mg/g tissue)
3.0
2.0
1.0
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

lactation ($p < 0.01$).

Free and esterified cholesterol responded differently during lactation in the myo-inositol deprived liver (Figure 2). A 50-fold increase in esterified cholesterol was observed for deprived animals when compared with supplemented animals, reaching this maximum by 21 days of lactation ($p < 0.01$), while no changes were observed for the supplemented animals during the course of lactation. Recovery of normal control (supplemented) levels of esterified cholesterol was apparent by four weeks after involution but required more than four days to occur (25 days post-birth). No significant changes were observed in liver free cholesterol between the two groups either due to dietary content of myo-inositol or lactation. Calculated recovery of lipid from both the deprived and supplemented animals as determined by summation of the four classes of lipid assayed ranged from 85% to 96% of the total lipid determined gravimetrically.

Electronmicroscopy. Electronmicrographs of the livers of dams fed a myo-inositol supplemented or deficient diet during lactation are shown in Figure 3. Electronmicrographs A and B are samples selected to be representative of the livers of those dams receiving the myo-inositol supplemented diet and C and D are those which represent animals on the deficient diet. The increase in the fat content of the liver of the deprived dam is reflected by an appropriate increase in the size and number of the fat droplets. In most of the electronmicrographs examined, a reduction in the amount of rough endoplasmic reticulum was noted for the deprived animals' livers, however, the significance of this qualitative observation remains undetermined.

Characterization of Plasma Lipids. An overall reduction in plasma lipids was observed during lactation for the deprived dams when

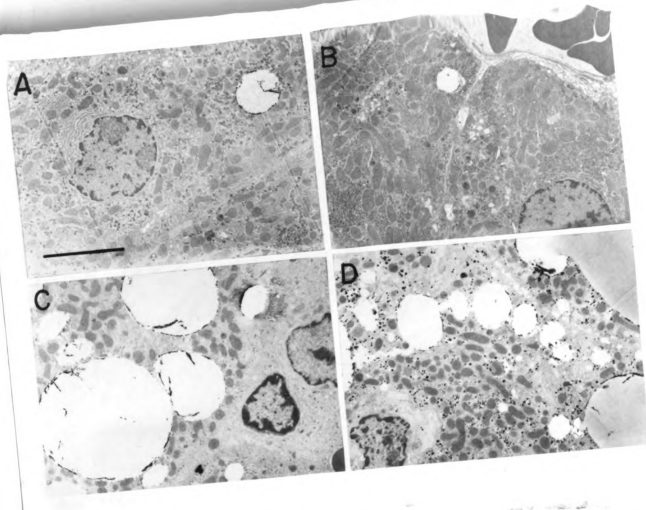


Figure 3. Electronmicroscopy of the Livers of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet at 14 Days of Lactation. Samples were prepared for microscopy as indicated in the Methods section. A and B represent samples from the livers of dams fed the supplemented diet. C and D represent samples from the livers of dams fed the deficient diet. Magnification is 4600 X for all the micrographs. The bar in A represents 5 um.

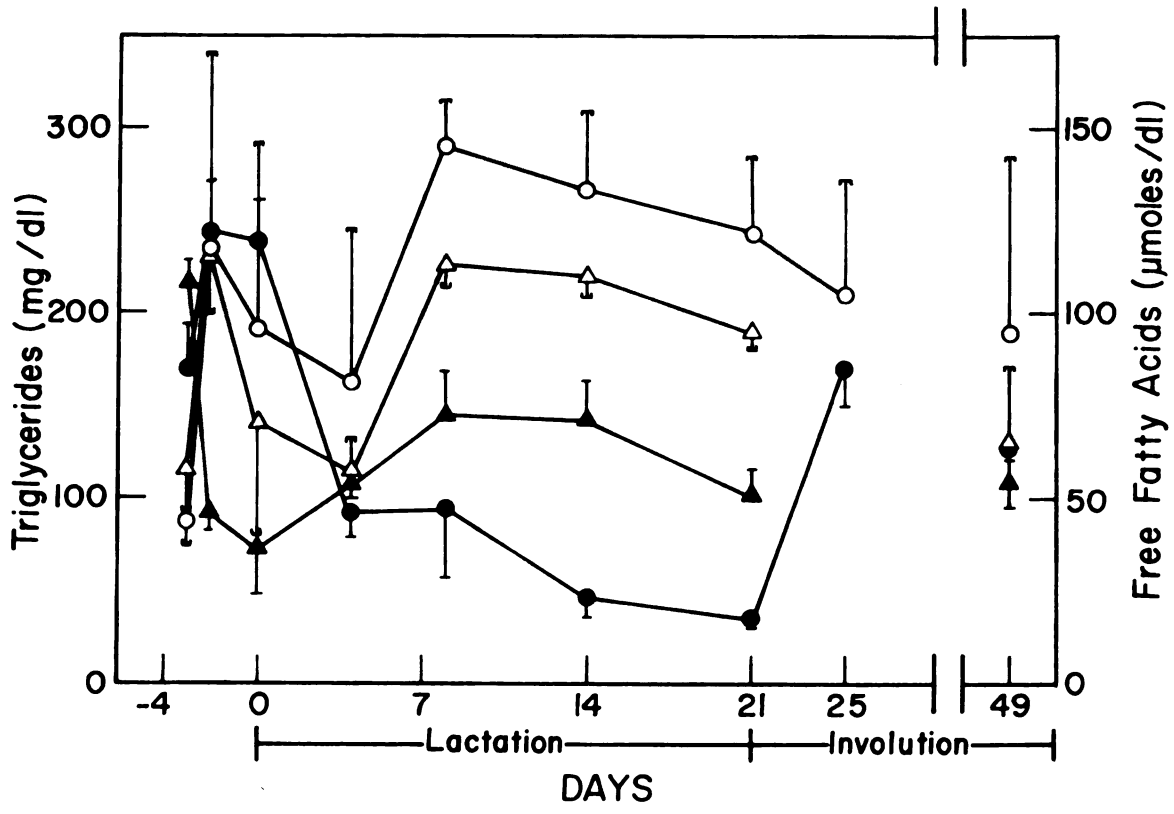
compared with the supplemented dams. Plasma triglycerides analysis (TG, Figure 4) revealed elevated levels of TG for the deprived dams above those observed for the supplemented dams at -2 ($p<0.001$) and 0 ($p<0.001$) days of age. Levels of TG at four and eight days of lactation were not significantly different for the two dietary groups. By 14 days of lactation, deprived animals showed significant ($p<0.005$) reduction in the triglyceride levels which were maintained through 21 days of lactation ($p<0.005$). Significant elevation ($p<0.005$) in plasma TG of deprived dams was observed during involution from weaning (21 days of lactation) to four days of involution (25 days after birth). Post-lactational plasma TG levels (four weeks of involution) showed no significant differences between supplemented and deprived dams.

During the course of gestation and lactation, plasma free fatty acid (FFA) levels of deprived and supplemented dams followed a parallel profile. The mean concentration of plasma FFA (Figure 4) increased at -2 days for both supplemented and deprived dams, declining steadily for both groups afterward, until four days of lactation. Increases in the mean FFA concentration were again observed for both groups from four to eight days of lactation which subsequently declined as lactation progressed. Although higher mean concentrations of plasma FFA were observed for deprived dams during the period from birth to four days of involution the levels were not statistically significant when compared with the levels observed in the plasma of supplemented dams.

Plasma free cholesterol (C, Figure 5) of myo-inositol deprived dams displayed significant reductions ($p<0.025$ at four days to $p<0.005$ at 14 days) when compared to supplemented animals throughout the period of lipid deposition in the liver whereas esterified cholesterol

Figure 4. Triglycerides and Free Fatty Acids in the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as mg of triglyceride/ dl (▲ , supplemented dams; ● , deprived dams) or umoles of free fatty acids/ dl (△ , supplemented dams; ○ , deprived dams). Values were not determined for supplemented animals 25 days after birth.

Figure 5. Free and Esterified Cholesterol Content of the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as mg of free (△ , supplemented dams; ○ , deficient dams) or esterified (▲ , supplemented; ● , deficient dams) cholesterol/ dl plasma. Values were not determined for supplemented animals 25 days after birth.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300
200
100
0
Free Fatty Acids (μmoles/dl)
150
100
50
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

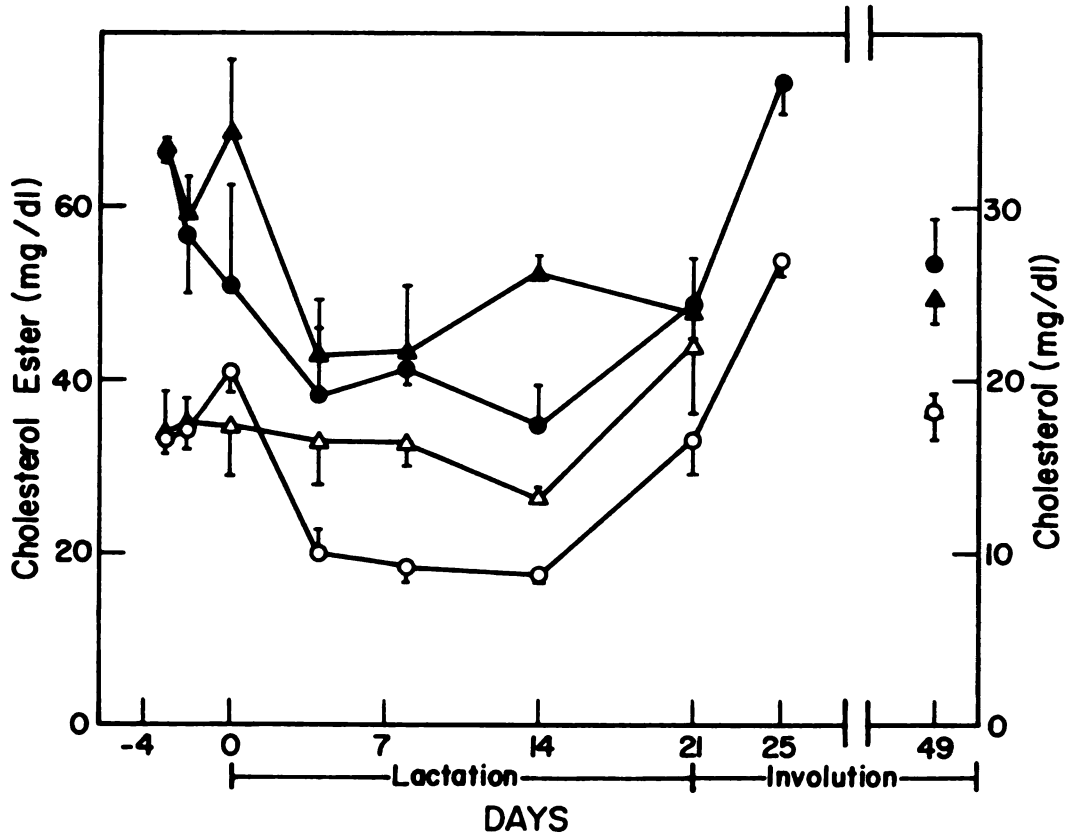
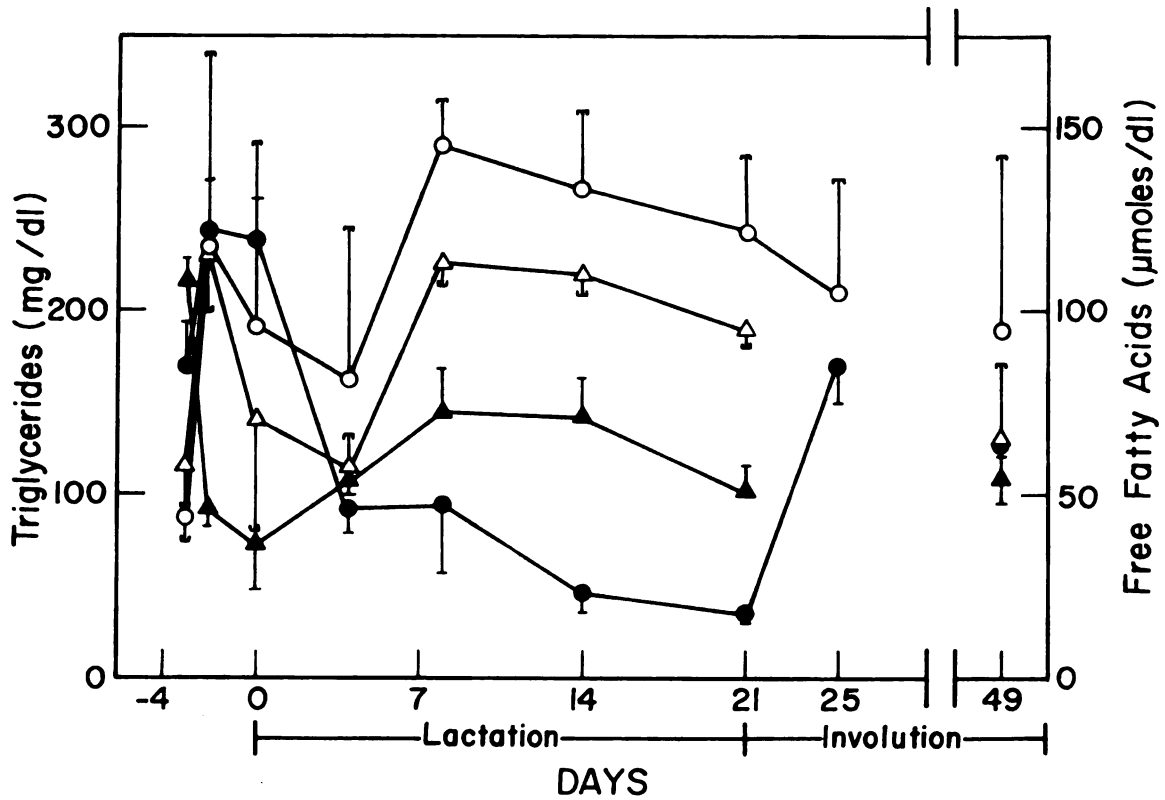
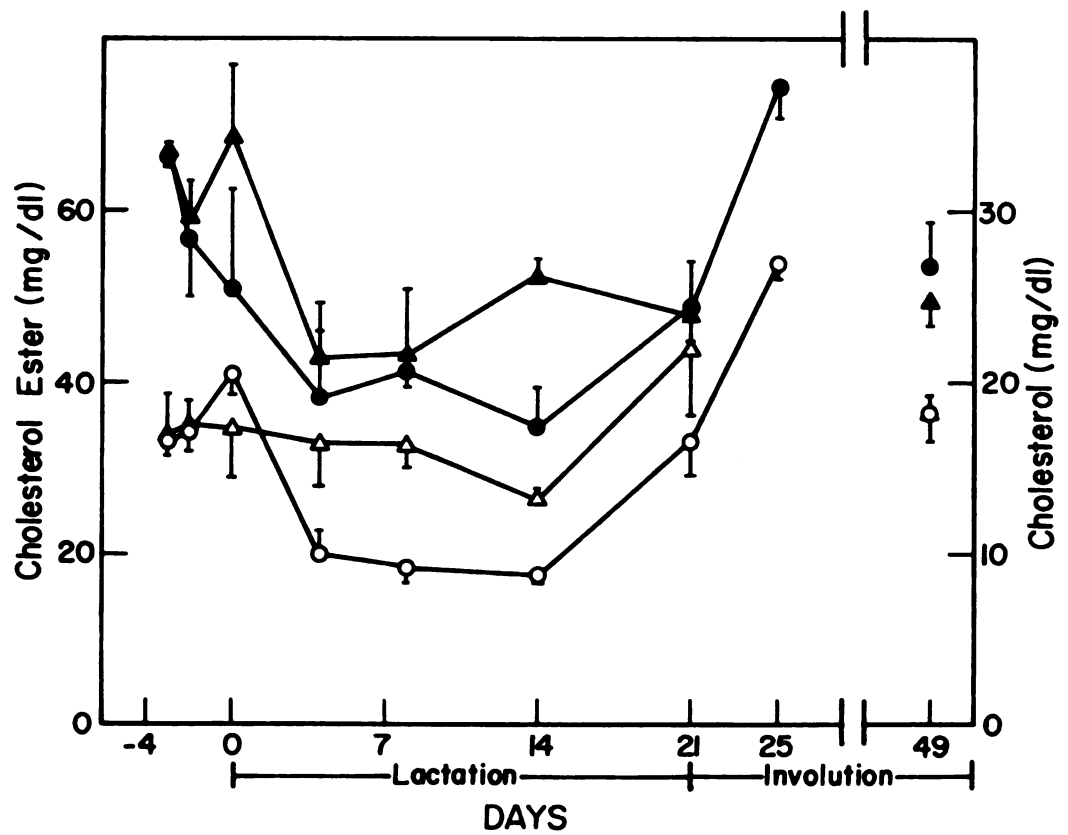

Cholesterol Ester (mg/dl)
60
40
20
0
Cholesterol (mg/dl)
30
20
10
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

Figure 4. Triglycerides and Free Fatty Acids in the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as mg of triglyceride/ dl (▲ , supplemented dams; ● , deprived dams) or umoles of free fatty acids/ dl (△ , supplemented dams; ○ , deprived dams). Values were not determined for supplemented animals 25 days after birth.

Figure 5. Free and Esterified Cholesterol Content of the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as mg of free (△ , supplemented dams; ○ , deficient dams) or esterified (▲ , supplemented; ● , deficient dams) cholesterol/ dl plasma. Values were not determined for supplemented animals 25 days after birth.


Triglycerides (mg/dl)
Free Fatty Acids (μmoles/dl)
300
200
100
0
150
100
50
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

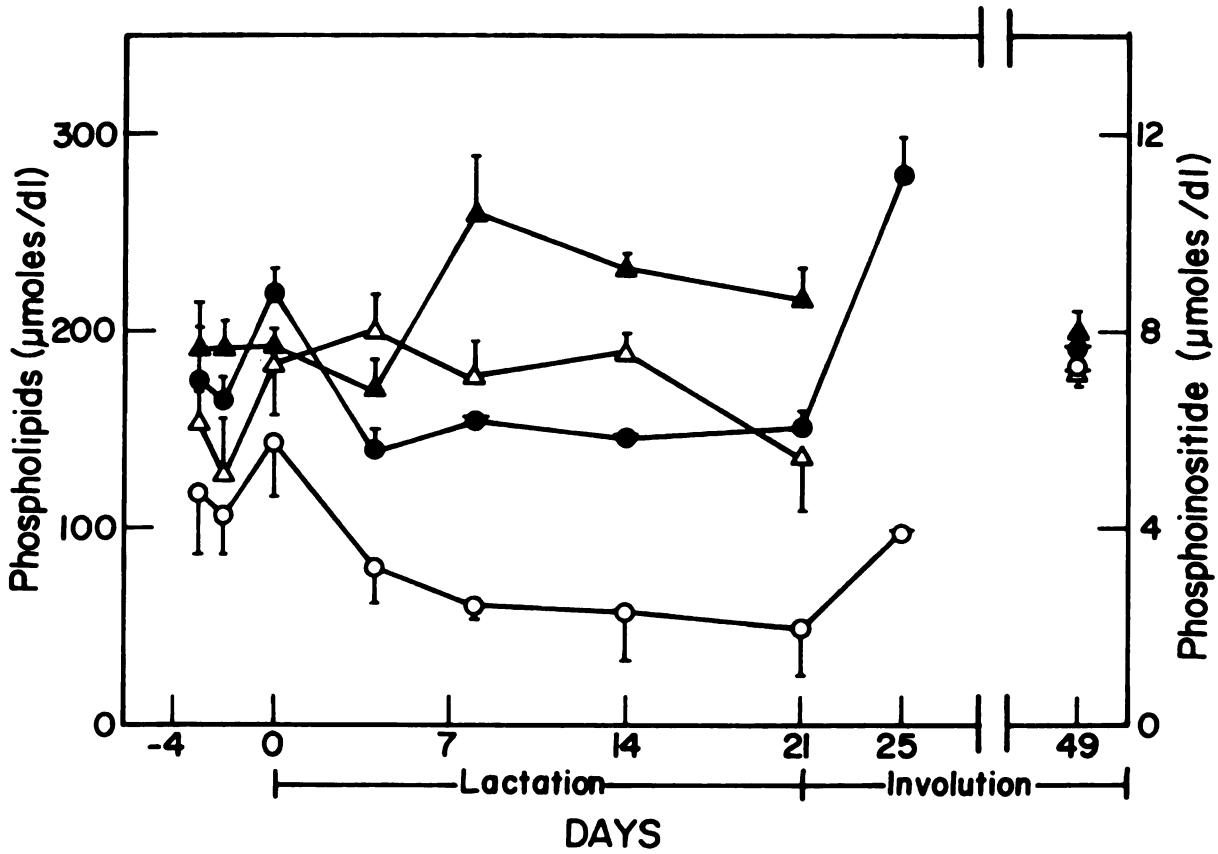

Cholesterol Ester (mg/dl)
Cholesterol (mg/dl)
60
40
20
0
30
20
10
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

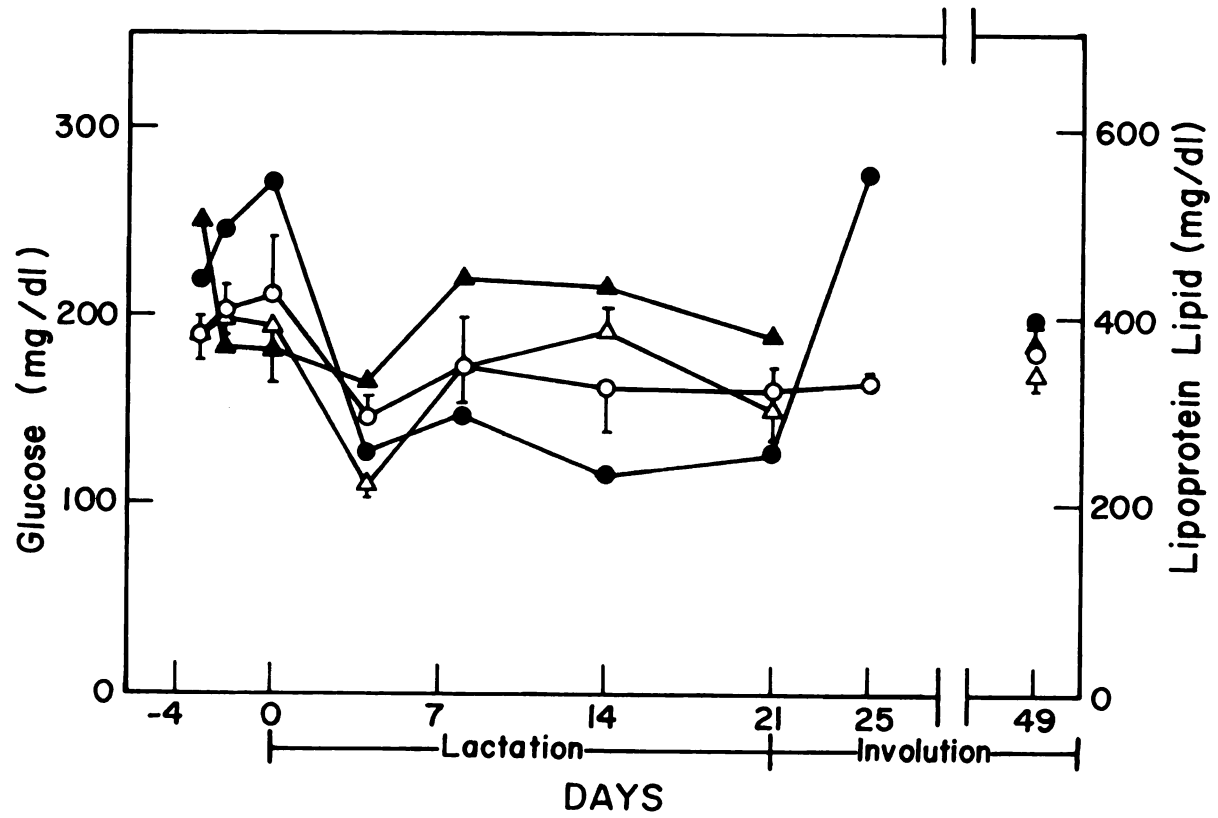
(CE, Figure 5) was significantly ($p<0.01$) reduced only at 14 days of lactation. Elevations in both free and esterified plasma cholesterol of deprived dams occurred during the involution period (four days post-weaning) but returned to lactational or pre-lactational levels by four weeks post-weaning. Phospholipids (PL) in the plasma (Figure 6) responded similarly to free cholesterol with significant reductions at 4 ($p<0.005$), 8 ($p<0.025$), 14 ($p<0.025$), and 21 ($p<0.02$) days of lactation. Phosphatidylinositol (PI) levels in the plasma paralleled the responses of total phospholipids during the lactation period, showing a more significant decrease for deprived dams (4 days, $p<0.005$; 8 days, $p<0.005$; 14 days, $p<0.005$; 21 days, $p<0.025$) throughout the lactation period. Elevated levels of both phosphatidylinositol and phospholipids were observed four days into involution followed by a return to pre-lactational levels by four weeks after weaning.

Figure 7 presents the effects of lactation and dietary myo-inositol on plasma glucose and lipoprotein-associated lipid levels (as determined by calculation: see Methods section). No consistent pattern or change in plasma glucose was observed as a result of feeding the myo-inositol supplemented or deficient diet during gestation (-3 days to birth), lactation (birth to 21 days of lactation) or involution (up to four weeks after weaning). Gestational plasma lipid levels in deprived dams were lower than supplemented dams at -3 days, abruptly changing at -2 days and reaching the maximum lipoprotein-associated lipid levels were consistently lower in the myo-inositol deprived dams with respect to the levels of the supplemented dams after the onset of lactation. Involution levels (four days after weaning) of plasma lipid in deprived dams were elevated above those determined for fully recovered dams of either diet at four weeks post-weaning.

Figure 6. Phospholipid and Phosphatidylinositol Content of the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. for 4 animals except 25 days after birth where n=3. Values are expressed as umoles of phospholipid/ dl plasma (▲ , supplemented dams; ● , deprived dams) or umoles of phosphatidylinositol/ dl plasma (△, supplemented dams; ○ , deprived dams). Values were not determined for supplemented animals 25 days after birth.

Figure 7. Glucose and Lipoprotein-Associated Lipid Content of the Plasma of Lactating Dams Fed a myo-Inositol Supplemented or Deficient Diet. Each point represents the mean ± S.D. (for glucose levels) for 4 animals except 25 days after birth where n=3, or the sum of the mean values of each of the 4 lipid classes (TG, C, CE, PL) of plasma representing lipoproteins for 4 animals except 25 days after birth where n=3. Values are expressed as mg/ dl glucose (△ , supplemented dams; ○ , deprived dams) or mg of lipoprotein-associated lipid/ dl (▲ , supplemented dams; ● , deprived dams). See the Methods section for factors used in converting lipid values to mg. Values were not determined for supplemented animals 25 days after birth.


Phospholipids (µmoles/dl)
Phosphoinositide (µmoles/dl)
300
200
100
0
12
8
4
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS


Glucose (mg/dl)
Lipoprotein Lipid (mg/dl)
300
200
100
0
600
400
200
0
-4
0
7
14
21
25
49
Lactation
Involution
DAYS

myo-Inositol Deprivation and Liver Lipid in Lactating Dams Fed a myo-Inositol Deficient Diet Without Sulfa. Lactating dams fed a myo-inositol deficient diet with the omission of an antibacterial drug (phthalylsulfathiazole) were observed to have normal appearing livers with lipid contents of 0.049 ± 0.012 g lipid/g fresh weight tissue (mean ± S.D., n=4).

myo-Inositol Deprivation and Kidney and Intestine Lipid Levels. In view of the reported effects of myo-inositol deprivation on intestinal lipid content in the gerbil (1, 2) and potential effects in the kidney, the lipid contents of intestine and kidney were examined in lactating dams. No significant effects were observed on the lipid content (mean ± S.D., n=4) of either the kidney (supplemented dams, 0.041 ± 0.003 g lipid/g tissue; deprived dams, 0.042 ± 0.007 g lipid/g tissue) or the intestine (supplemented dams, 0.048 ± 0.006 g lipid/g tissue; deprived dams, 0.049 ± 0.006 g lipid/g tissue) of supplemented versus deprived 21 day lactating rats.

DISCUSSION

Although the lipotropic action of myo-inositol has been discussed for a number of years (1 - 4, 12 - 18) the question of its mechanism remains unanswered. Conflicting results in work with myo-inositol deprivation (12 - 18) has resulted in the difficulty of associating a consistent lipotropic action with the polyol. Recent work (1 - 4) has again stimulated study of the lipotropic effects of myo-inositol with an emphasis on the interaction of myo-inositol deprivation and dietary fats (1 - 3, 19). The work of Hegsted's group (1, 2) has shown the dependence of intestinal lipodystrophy in female gerbils on myo-inositol

deprivation and saturated fats in the diet. Hayashi et al. (3) have also demonstrated a fatty liver with similar requirements in the male weanling rat. Andersen and Holub (19), however, have shown that of four dietary fats tested, the most unsaturated fat (Tower rapeseed oil) also produced the syndrome of fatty liver in male weanling rats. The work of Burton and Wells (Chapter III, 4) with a myo-inositol deficient diet containing an unsaturated fat (soybean oil) showed an insignificant development of the syndrome in the developing rat of both sexes while at the same time producing a severe fatty liver in lactating dams. These data suggest that saturation of the fat cannot be the only factor which determines the development of fatty liver during myo-inositol deprivation.

Although the mechanism of the accumulation of lipid in the liver or intestine of animals fed myo-inositol restricted diets is largely unknown, many investigators (2, 4, 20) have suggested the inhibition of lipoprotein formation or secretion as the major cause.

Phospholipids (PL) including phosphoinositides (PI) are known to be involved in the synthesis of lipoproteins (holo form) in the liver (22 - 24). Reductions in the levels of liver PL and PI (Chapter III, Figure 5C) might be expected to interfere with the normal synthesis of holo-LP and result in the reduction of plasma lipids associated with LP. Of the lipids examined (TG, C, CE, and PL) which are known to be associated with LP synthesis in the liver, phospholipid (PL) was the only lipid class shown to be decreased (Figure 1, 2), thus potentially interfering with holo-LP synthesis.

No evidence has been obtained showing interference of apo-LP synthesis by myo-inositol deprivation. In addition, myo-inositol deprived

fatty livers (3, 4, 19) have shown to have characteristics similar to those of choline deficient fatty livers (25, 26) for which apolipoprotein synthesis is reported to be normal (22). However, inhibition of apo-LP synthesis cannot be ruled out for myo-inositol deprived fatty livers.

Hayashi et al. (21) have considered excessive mobilization of free fatty acids (FFA) from adipose depots (possibly controlled by levels of myo-inositol) to be the more direct cause of fatty liver syndrome in myo-inositol deficiency. Our results have suggested the mechanism to be inhibition of LP secretion by the liver because of the overall reduction in plasma lipids known to be associated with LP. However, the mechanism proposed by Hayashi et al. (21) cannot be ruled out in myo-inositol deprived, lactation-induced fatty liver since elevation of the mean plasma FFA concentration above control values was observed for myo-inositol deprived dams during lactation.

The role which hormones play in the production of the fatty liver syndrome during myo-inositol deprivation has not been examined. Epinephrine and norepinephrine are known to stimulate levels of plasma FFA and subsequently the levels of plasma TG (27). A variety of other hormones are also known to stimulate or repress the metabolism of LP (22, 23). A combined mechanism of plasma FFA elevation via hormonal influence coupled with myo-inositol deprivation and its subsequent effects on PL and PI may be adequate to interfere with normal LP metabolism during lactation. This suggested mechanism resembles the Type II fatty liver discussed by Scanu (23) with the modification of elevated plasma FFA and has its basis in the observed increases in plasma FFA (3, 4), the reduction of liver free and lipid-bound myo-inositol concurrent with liver lipid deposition and lactation,

subsequent reduction in lipoprotein associated lipids of plasma and return to normal liver and plasma lipid levels after the cessation of lactation.

Finally, alterations in PL metabolism during myo-inositol deprivation may possibly affect microsomal and/or cellular membranes such that normal LP secretion is impaired. Parallel observations during choline deficiency (28) have been suggested as an explanation for reduced or impaired release of plasma proteins other than LP.

While no dramatic changes in the liver such as lipid deposition occurred during gestation, significant elevation in plasma lipids was observed in both groups. The elevation in plasma lipids for deprived animals over that of the supplemented animals was in direct opposition to that observed during lactation. This might suggest a significant role for myo-inositol, such as control of FFA mobilization (21), in reducing the hyperlipemia that has been observed during the latter portion of gestation for several animal species including the rat and man (29, 30).

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CHAPTER V

myo-INOSITOL IN THE DEVELOPING RAT BRAIN: BIOSYNTHETIC ENZYMES AND DIETARY EFFECTS ON FREE AND LIPID-BOUND myo-INOSITOL AND FUMARASE AND 2', 3'-CYCLIC NUCLEOTIDE-3'-PHOSPHOHYDROLASE ACTIVITIES

ABSTRACT

myo-Inositol and its biosynthetic enzymes were examined during prenatal and postnatal development in the rat. Brain levels of free myo-inositol revealed a pattern very similar to that reported elsewhere (1, 2). L-myo-Inositol-1-phosphate synthase (EC 5.5.1.4) showed a sharp threefold rise in specific activity after parturition followed by a continual decline to 50% of the prepartum activity throughout the time course studied. L-myo-Inositol-1-phosphate phosphatase (EC 3.1.3.25), however, revealed nearly a twofold rise after birth which remained constant until 12 days of age and decreased to 150% of the prepartum activity by 21 days of age.

The effects of myo-inositol deficieney on brain were also examined during neonatal development in the rat. Neonatal rats, six days of age, were fed a myo-inositol deficient liquid formula by gastric intubation for ten days, after which they were fed a purified myo-inositol free diet until they were 72 days old. The free and lipid-bound myo-inositol levels of cerebrum and the free myo-inositol of the cerebellum were unaffected when comparing supplemented with deficient diet fed animals. The cerebrum and cerebellum of myo-inositol deficient rats had normal myelination and mitochondriogenesis as judged by comparison of the levels of 2', 3'-cyclic nucleotide-3'-phosphohydrolase

(EC 3.1.4.1) and fumarase (EC 4.2.1.2) activity respectively, with those of the supplemented animals.

INTRODUCTION

Studies regarding myo-inositol and growth generally deal with the weanling animal (3 - 5). In addition, attention has usually been focused on overall body growth (4, 5) alteration of lipid metabolism (6), and alopecia (5). Rapidly proliferating mammalian cell lines have been shown to require myo-inositol for in vitro growth (7, 8) as well as several strains of yeast which require myo-inositol in spite of possessing enzymes of the biosynthetic pathway (9, 10). Recent work has also shown that rat and human milk contain a rich supply of myo-inositol (11). These studies suggest that immature mammals may require an exogenous source of myo-inositol for normal growth and development.

In particular, work involving the role myo-inositol plays in neural tissue has been limited to its developmental levels in brain (Chapter III, 1, 2), a study on impulse velocities and myo-inositol levels in nerves (12) and myo-inositol in normal versus degenerating nerves (13).

This work focuses on the central nervous system and examines exogeneous and endogenous sources of myo-inositol and their effects on the development of rat brain with respect to tissue levels of myo-inositol, mitochondriogenesis and myelination.

MATERIALS AND METHODS

Animals and Diets. For the enzymatic studies, timed pregnant female rats of the Holtzman strain (Madison, Wisconsin) weighing

250-300 g were used. Females were fed a commercial stock diet[1] and water ad libitum. Pups were allowed to nurse normally and weaned at 21 days of age. All litters were maintained at eight pups per dam.

For the dietary studies, day old rat pups of the Holtzman strain (Madison, Wisconsin) were distributed randomly into two groups of eight litters of eight pups each. The pups were kept on wood shavings in polycarbonate cages occupied by a non-lactating foster dam that had recently (four to six weeks) nursed a litter. Foster mothers were fed a commercial stock diet[1] and water ad libitum.

The pups were fed a liquid formula by stomach tube according to a modification of the technique of Miller and Dymsza (14) with the composition reported by Dymsza, et al. (15). The formula contained (% by weight): non-fat dry milk, 15.0; corn oil, 10.0; myo-inositol-free vitamin mix, 1.0; choline chloride, 0.2; calcium carbonate, 0.9; and water, 72.0. To the control formula, 100 mg of myo-inositol were added per 100 ml of formula at the expense of water. The intubation was accomplished with appropriate lengths of polyethylene tubing (intramedic PE-50, Clay Adams, Inc.) attached to a blunted size 22 gauge needle fitted on a 1 or 2 ml glass syringe. Each animal was fed approximately 0.03 ml formula per gram body weight every four hours. Formulae were shown by gas-liquid chromatography (16) to contain 7.44 ± 0.14 mg/100 ml and 114.8 ± 1.7 mg/100 ml of myo-inositol for the deficient and supplemented diets, respectively.

Pups were weaned at 16 days of age and fed either a purified diet deficient in myo-inositol, or an identical diet supplemented with

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[1] Lab-Blox, Allied Mills, Inc., Chicago, Illinois

250 mg myo-inositol per 100 g diet at the expense of sucrose. The purified diet consisted of the following (% by weight): sucrose, 64.9; casein, 25.0; Wesson salt mix[2], 4.0; soybean oil, 4.0; choline chloride, 0.1; and vitamin mix[3], 1.0.

Tissue Preparation. Tissues were obtained from ether anesthetized animals in each experimental group at selected ages from three days prepartum to 72 days postpartum. Blood was removed by cardiac puncture into chilled heparinized tubes and plasma samples were prepared by centrifugation and stored at -80° C until free myo-inositol levels were determined (16). Brain tissue was quickly removed, chilled on crushed ice, weighed and stored at -80° C until subsequent analysis of free and lipid-bound myo-inositol (16, 17) or free myo-inositol and the activities of the myo-inositol synthesizing enzymes (11). Cerebral and cerebellar samples from the dietary studies were assayed for 2', 3'-cyclic nucleotide-3'-phosphohydrolase (EC 3.1.4.1), a myelin marker enzyme, by the method of Prohaska, et al. (18) and fumarase (EC 4.2.1.2) was measured by the method of Racker (19) after treatment of the tissue with Triton X-100, 0.5% (v/v).

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[2]Wesson Salt Mixture, ICN Nutritional Biochemicals, Cleveland, Ohio. The salt mix consisted of (%): $CaCO_3$, 21.00; $CuSO_4$ $5H_2O$, 0.039; $FePO_4$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.005; NaCl, 10.500; NaF, 0.057, and $Ca_3(PO_4)_2$, 14.900

[3]Vitamin fortification mixture, ICN Nutritional Biochemicals, Cleveland, Ohio. The vitamin mix furnished per 100 g of diet (mg): α-tocopherol, 5.0; ascorbic acid, 45; choline chloride, 75; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine-HCl, 1.0; thiamin-HCl, 1.0; Ca-pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin B-12, 0.0014; vitamin A, 900 IU; and vitamin D_3, 100 IU.

RESULTS

Free myo-Inositol in Developing Rat Brain. Figure 1 shows the free myo-inositol content of developing rat pup cerebrum where the respective dams were fed a commercial pellet diet. The highest levels (4.4 umoles/g tissue), during the time course studied, were observed for fetal rats at two days prepartum after which a decrease in the levels occurred (2.5 umoles/g tissue at eight days of age). This minimal level was maintained until 16 days of age increasing again to 3.0 umoles/g tissue by weaning. This pattern of free myo-inositol closely resembled that reported in previous work (Chapter III) and by other investigators (1, 2).

Developmental Profile of Rat Brain myo-Inositol Synthase and L-myo-Inositol-1-Phosphate Phosphatase. A developmental profile of the myo-inositol biosynthetic enzymes for rat brain is shown in Figure 2.

Early fetal brain (-4 days to birth) showed a reduction in synthase activity prior to birth. A subsequent rise in synthase activity was observed after birth to one day of age (13 m units[4]/mg protein) followed by a steady decrease to a minimal activity (3 m units[4]/mg protein) at 21 days of age. After a plateau level of 0.6 units[4]/mg protein during the prepartum period, the phosphatase developmental profile for brain (Figure 2) showed an elevation in activity to approximately 1.1 units[4]/mg protein during the first half of the neonatal period. This activity level decreased slowly to 0.9 units[4]/mg protein from 12 to 21 days of age.

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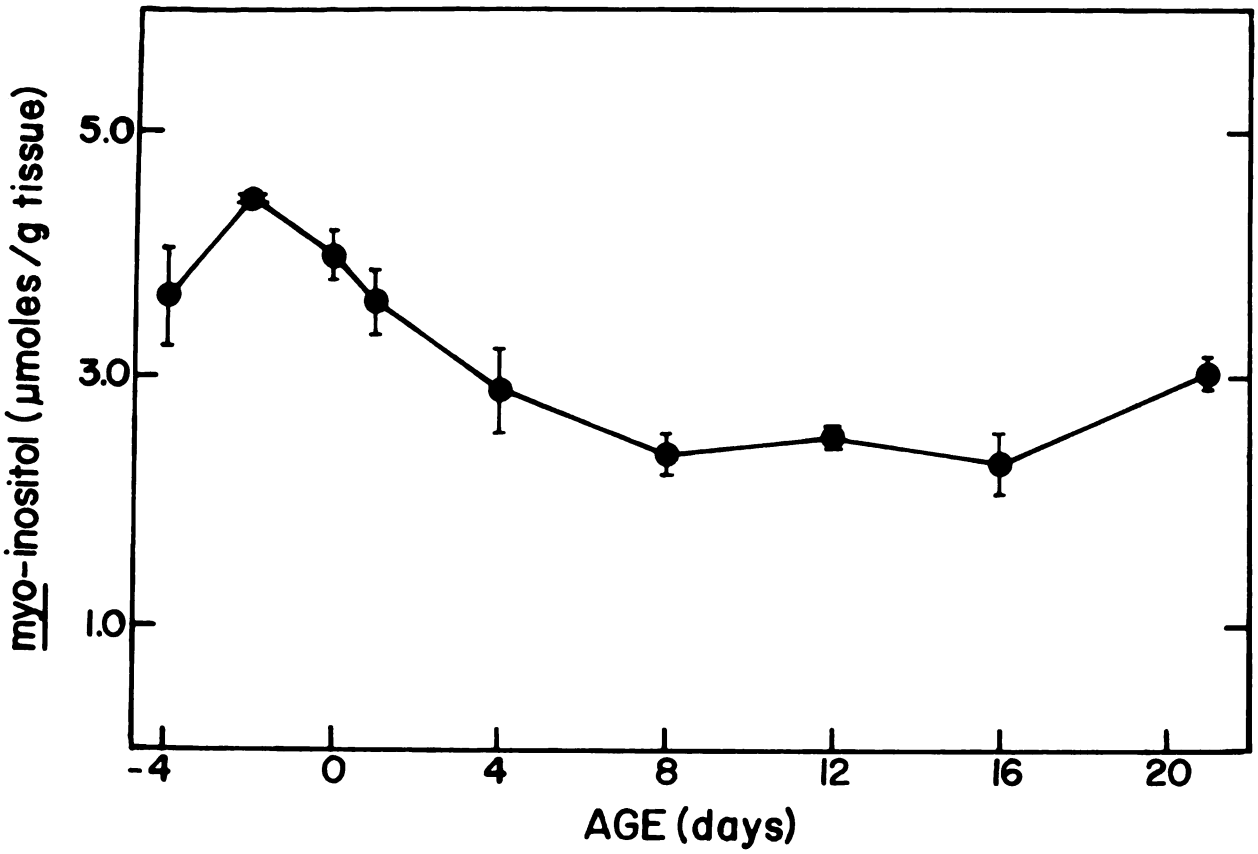
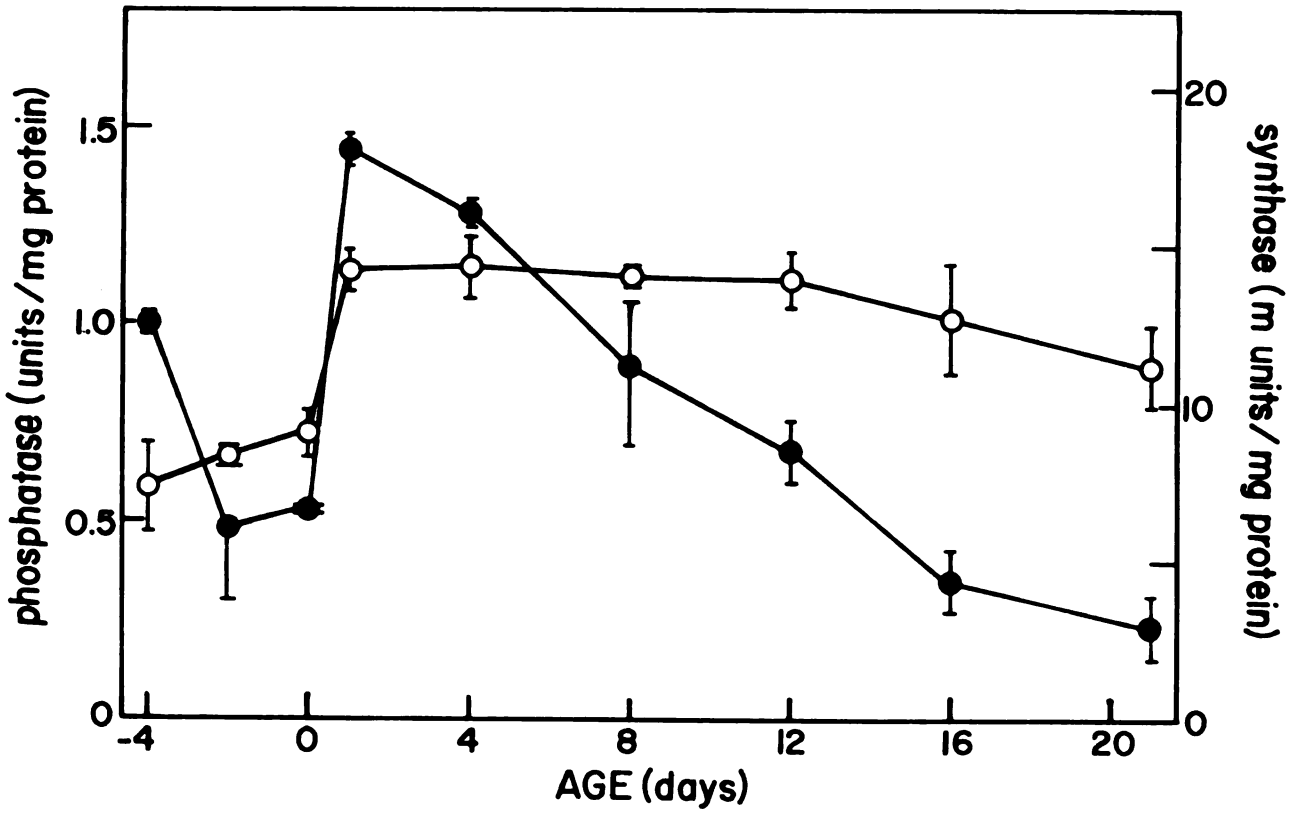
[4]A unit of activity represents 1 umole of myo-inositol-1-phosphate synthesized (synthase) or hydrolyzed (phosphatase) per hour. 1 m unit = 0.001 units

Figure 1. Free myo-Inositol Content of Developing Rat Brain. Pups were nursed by dams fed a commercial pellet diet. Each value is the mean ± S.D. for 4 pools of 8 animals for neonates or for 4 pools of fetuses (each pool from 1 dam).

Figure 2. L-myo-Inositol-1-Phosphate Synthase and L-myo-Inositol-1-Phosphate Phosphatase Activities in Rat Brain During Development. Pups were nursed by dams fed a commercial pellet diet. Each value is the mean ± S.D. for 4 pools of 8 animals for neonates or 4 pools of fetuses (each pool from 1 dam). See footnote 4 for definition of activities. Synthase (●), phosphatase (○).


myo-inositol (μmoles/g tissue)
5.0
3.0
1.0
-4
0
4
8
12
16
20
AGE (days)


phosphatase (units/mg protein)
1.5
1.0
0.5
0
synthose (m units/mg protein)
20
10
0
-4
0
4
8
12
16
20
AGE (days)

Adult synthase (2.7 ± 0.3 m units/mg protein, n=4) and phosphatase (0.62 ± 0.20 units/mg protein, n=4) activities do not significantly vary from those of the weanling pup (21 days).

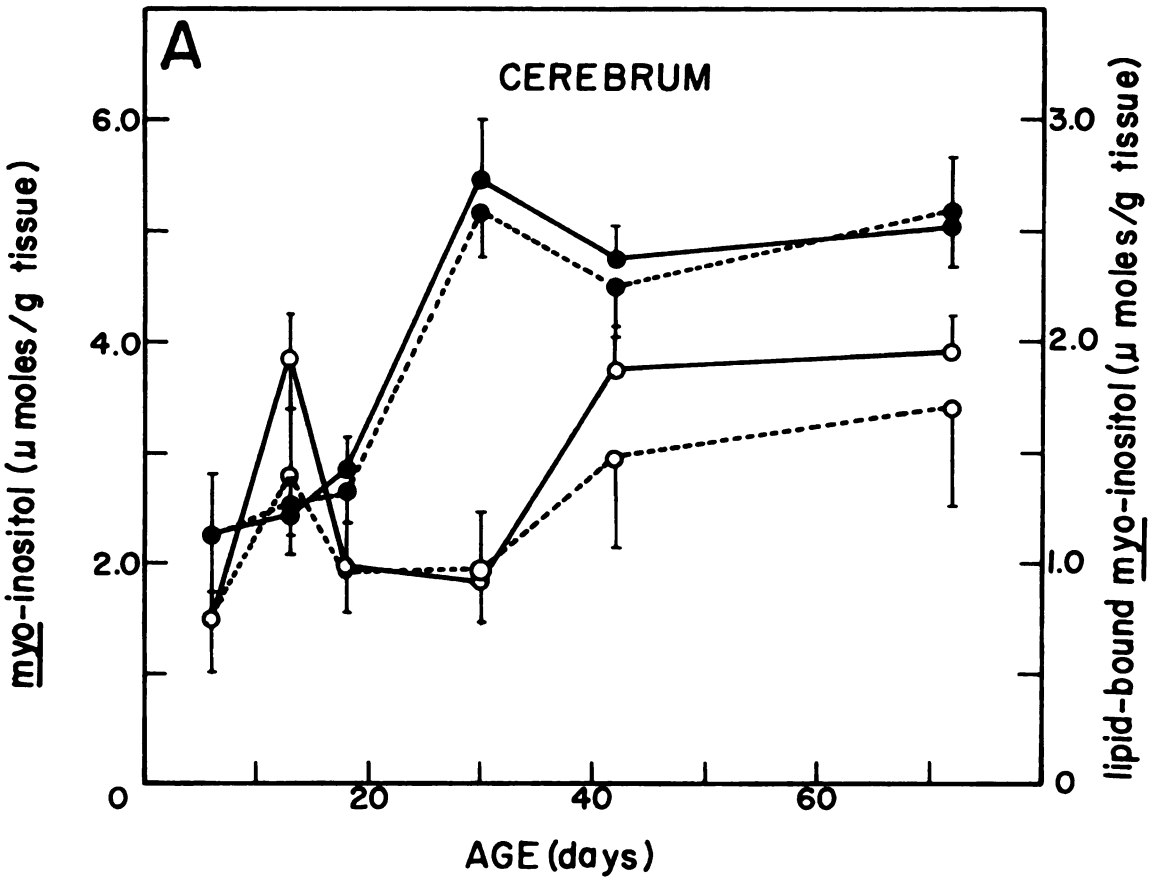
Effects of Dietary myo-Inositol on myo-Inositol Levels in Neural Tissues. myo-Inositol levels of neural tissues were generally unaffected by nutritional myo-inositol deficiency. Figure 3 shows free and lipid-bound myo-inositol in developing rat cerebrum. No significant differences were observed in either free or lipid-bound myo-inositol when comparing the supplemented and deficient diet fed pups. In addition, developmental free myo-inositol levels paralleled those previously reported (Chapter III, 1, 2) and lipid-bound levels tended to reflect the tissue free pool size. Cerebellar free myo-inositol (Figure 4) revealed a developmental pattern similar to that displayed by cerebrum, however, the free pool levels were generally 20% higher than cerebral values.

Central Nervous System Myelination and Mitochondriogenesis. A comparison was made of an enzyme preferentially associated with myelin, 2', 3'-cyclic nucleotide-3'-phosphohydrolase, and an enzyme associated with mitochondria, fumarase, from cerebral and cerebellar tissues between neonatal rats fed myo-inositol supplemented or deficient diets. Although at 13 and 30 days of age some differences were observed in the phosphohydrolase activity of cerebellum, no consistent differences were observed in the activities of either enzyme as a result of dietary myo-inositol deficiency (Figures 4, 5).

DISCUSSION

Of the variety of tissues which have been examined in the rat, neural tissues rank among the highest in tissue content of free

Figure 3. Free myo-Inositol (●) and Lipid-Bound (○) myo-Inositol Content of A) Cerebrum and B) Cerebellum From Rats Fed a myo-Inositol Supplemented or Deficient Diet. Each value is the mean ± S.D. for four animals. Supplemented diet fed animals (————), deficient diet fed animals (- - - - -).


A
CEREBRUM
6.0
4.0
2.0
0
3.0
2.0
1.0
0
20
40
60
AGE (days)
myo-inositol (μ moles/g tissue)
lipid-bound myo-inositol (μ moles/g tissue)

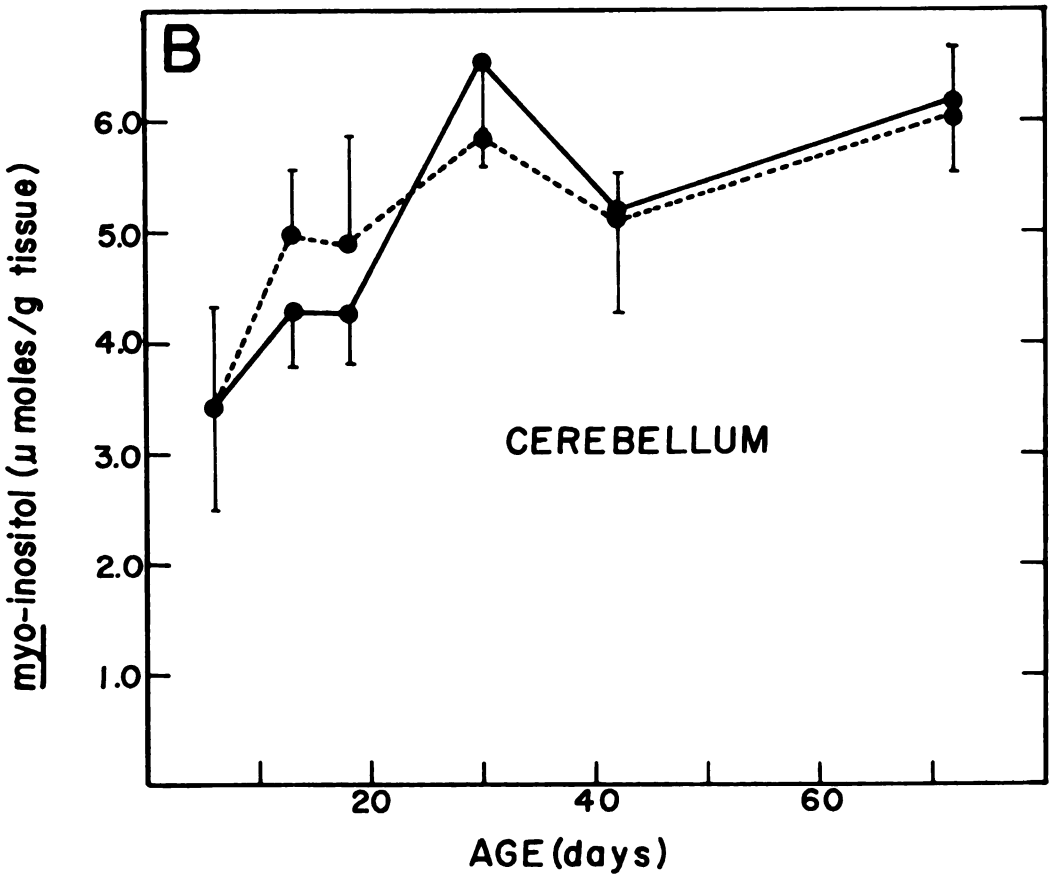
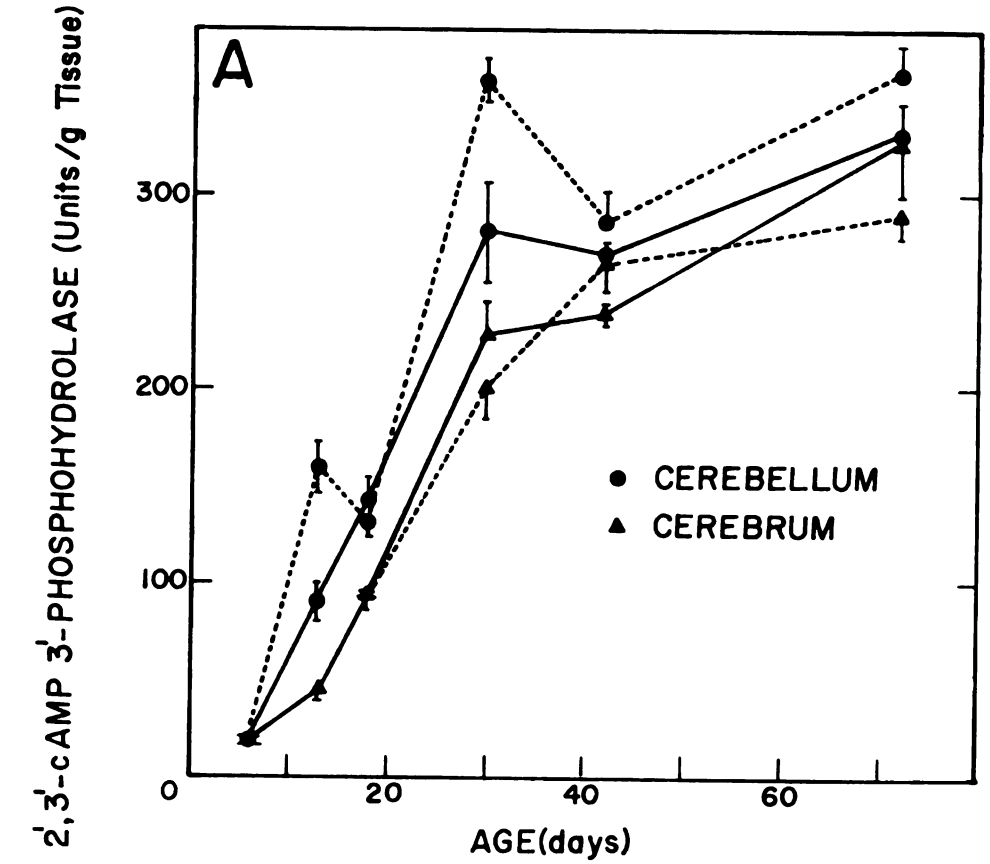
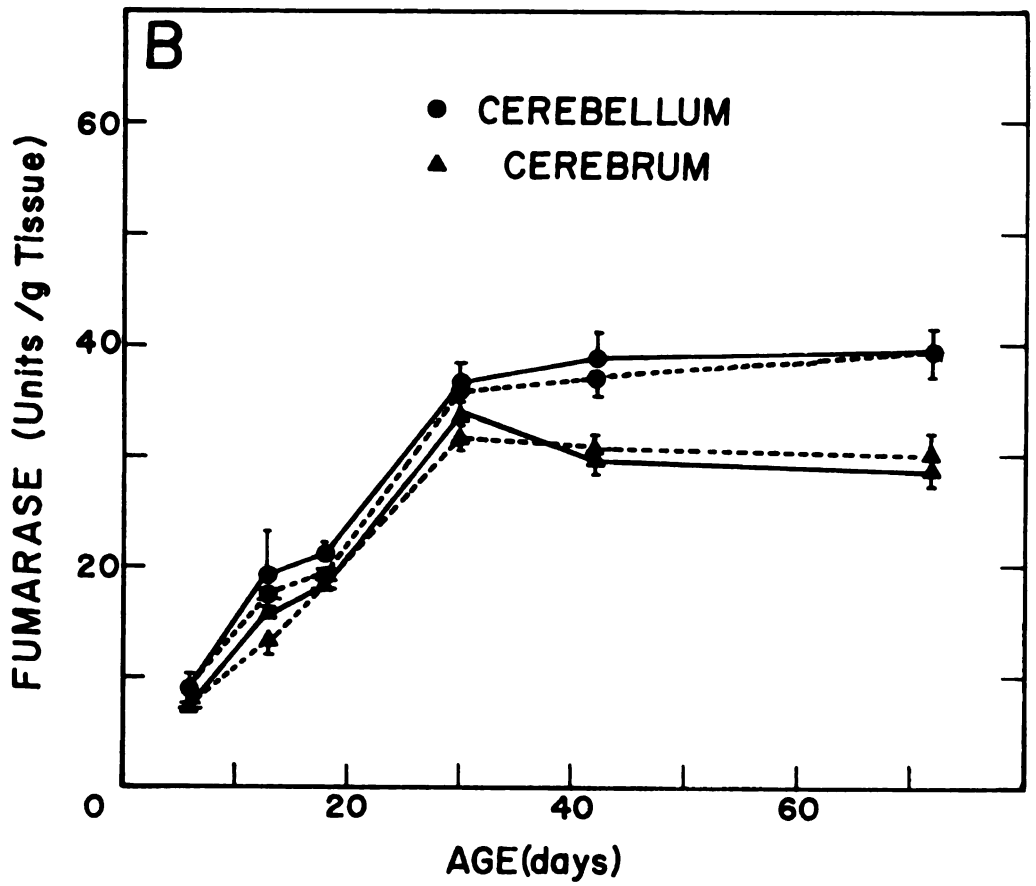

B
6.0
5.0
4.0
3.0
2.0
1.0
CEREBELLUM
20
40
60
AGE (days)
myo-inositol (μ moles/g tissue)

Figure 4. Comparison of Cerebellar (●) and Cerebral (▲) A) 2', 3'-c-AMP-3'-Phosphohydrolase and B) Fumarase from Rats Fed a myo-Inositol Supplemented or Deficient Diet. Each value is the mean ± S.D. for four animals. Supplemented diet fed animals (———), deprived diet fed animals (- - - -).


A
2',3'-cAMP 3'-PHOSPHOHYDROLASE (Units /g Tissue)
300
200
100
0
20
40
60
AGE(days)
CEREBELLUM
CEREBRUM


B
FUMARASE (Units /g Tissue)
60
40
20
0
20
40
60
AGE(days)
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myo-inositol (Chapters I and III). The question of the origin of brain myo-inositol, however, remains unclear. Optional sources of myo-inositol available in brain which might contribute to alterations in the free pool of myo-inositol could include: in situ synthesis from glucose, transport from the plasma, isolated pool(s) of myo-inositol (potential degradation of the lipid-bound form), or increased cellular retention of myo-inositol.

The purpose of this investigation was to further examine the origin of brain myo-inositol with regard to the role played by the known biosynthetic pathway as opposed to an exogenous (plasma, diet) source. Earlier work has revealed the ability of the brain to synthesize myo-inositol from glucose (20) and to transport myo-inositol from the blood (21). Work involving galactose toxicity and myo-inositol metabolism (1) suggested that brain myo-inositol levels can be significantly affected by inhibition of the biosynthesis of myo-inositol. Developmentally, we have shown a reduction in the biosynthetic ability of the rat brain as judged by determination of enzyme activities, specifically the decline in the rate limiting synthase activity (Figure 2). This is in agreement with the work of Hauser and Finelli (20) who found higher synthetic activity in young than adult rats.

Previous experiments have revealed the inability to significantly affect the levels of brain myo-inositol in the rat by dietary regimen (Chapters II, III; Figures 3A, 3B). These data in addition to those in Chapter I and Figure 2 would suggest that changes in rat brain myo-inositol may be due to developmental differences in the tissue's ability to transport and/or retain myo-inositol intracellularly. This would explain the brief decrease in brain myo-inositol early in development of the neonatal rat, i.e., the biosynthetic ability decreased

while the retention/transport function increased. Dietary and biosynthesis studies, however, cannot rule out the previously suggested possibilities of more than one pool of brain myo-inositol with differential turnover rates (21). These data neither confirm nor deny this possibility, however, the resistance of older animals to changes in brain free myo-inositol with reduced biosynthetic capacity fed myo-inositol deficient diets might suggest a pool with significant immunity to plasma and biosynthetic variation.

Stewart et al. (22) have suggested that a large proportion of brain myo-inositol is located in the glial cells which are believed to be responsible for the synthesis and maintenance of myelin (23). Examination of the developmental profile of rat brain free myo-inositol content regardless of the myo-inositol dietary source (Chapters III and V, 2) shows a correlation between the increase in brain myo-inositol and the developmental period of myelination (2). Phosphoinositides, for which myo-inositol is a precursor, are believed to play an important role in the structure and function of myelin (24, 25). As indicated in Figures 3A and 3B, dietary myo-inositol did not appear to significantly affect the free or lipid-bound pools of myo-inositol in the developing rat brain. Furthermore, the ability of the immature central nervous system to maintain normal levels of free and lipid-bound myo-inositol despite dietary deficiency, correlated with a normal extent of myelination and mitochondriogenesis as determined by the levels of 2', 3'-cyclic nucleotide-3'-phosphohydrolase and fumarase activity, respectively.

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CHAPTER VI

STUDIES ON THE EFFECTS OF 5-THIO-D-GLUCOSE AND 2-DEOXY-D-GLUCOSE ON myo-INOSITOL METABOLISM IN MICE AND THE INHIBITION BY 5-THIO-D-GLUCOSE-6-PHOSPHATE OF L-myo-INOSITOL-1-PHOSPHATE SYNTHESIS, IN VITRO

ABSTRACT

5-Thio-D-glucose-6-phosphate has been shown to be a competitive inhibitor of the enzyme D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) in vitro. The K_m for D-glucose-6-phosphate was 0.51 mM and the K_i for 5-thio-D-glucose-6-phosphate (5TG-6-P) was 0.33 mM. 5TG-6-P proved to be a poor substrate of the D-glucose-6-phosphate dehydrogenase reaction (V_{max} of 5TG-6-P = 0.444 V_{max} of glucose).

A high dosage (250 mg/kg body weight/day for seven days) of 5-thio-D-glucose (5TG) administered intraperitoneally stimulated levels of free myo-inositol in testes and liver and D-glucose-6-phosphate in testes of mice. A lower dosage of 2-deoxy-D-glucose (2DG, 50 mg/kg body weight/day for seven days) was found to significantly reduce levels of free myo-inositol in plasma, testes, and liver as a function of time.

Both 5TG (250 mg/kg body weight) and 2DG (50 mg/kg body weight) were found to significantly reduce the sperm counts of males after three and seven days of administration.

INTRODUCTION

The biosynthesis of myo-inositol from D-glucose-6-phosphate is catalyzed by D-glucose-6-phosphate: L-myo-inositol-1-phosphate

synthase (synthase, EC 5.5.1.4) and L-myo-inositol-1-phosphate phosphatase (EC 3.1.3.25). The synthase has been shown to be the rate limiting step with a requirement for NAD^+ (1) and has been the most extensively studied of the two enzymes with respect to properties (2 - 4) and mechanism (5 - 8). The synthase has been classified as a cycloaldolase catalyzing an intramolecular oxidation-reduction sequence at C-5 utilizing NAD^+ and a Schiff's base intermediate (1, 4, 5). Characterization of this enzyme has produced several studies involving inhibitors, for example, pyrophosphate (9), NADH (3, 4) and substrate isomers (4) the most effective of which is 2-deoxy-D-glucose-6-phosphate (10).

The D-glucose analog, 5-thio-D-glucose, first synthesized by Feather and Whistler (11) in 1962, has been demonstrated to produce a variety of cytological effects including inhibition of insect larval development (12), diabetogenic action in rats (13), potential antitumorogenic action (14) and inhibition of spermatogenesis in mice (15). In rats, urinary excretion of 95% of a pharmacological dose of the thio-sugar occurred within six hours (13). We have investigated the possibility of 5-thio-D-glucose phosphorylation at the 6 position, *in vivo*. 5-Thio-D-glucose-6-phosphate might be expected to inhibit the biosynthesis of L-myo-inositol-1-phosphate from D-glucose-6-phosphate as an explanation for the inhibition of spermatogenesis (15). The basis for this possibility lies in the absolute growth requirement of myo-inositol in mammalian cells (16, 17), active biosynthesis of myo-inositol in testes, *de novo* (8), and the proposed ability of myo-inositol to counteract the antimitogenic activity of colchicine (18).

Therefore, the effect of intraperitoneal administration of

5-thio-D-glucose and 2-deoxy-D-glucose on myo-inositol and D-glucose-6-phosphate and ATP levels in the testes and liver of mice have been investigated. In addition, it has been demonstrated that 5-thio-D-glucose-6-phosphate, prepared by the action of yeast hexokinase on 5-thio-D-glucose and ATP, competitively inhibits D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) from rat testes.

MATERIALS AND METHODS

Materials. All reagents used were analytical grade. Yeast hexokinase, yeast glucose-6-phosphate dehydrogenase, E. coli alkaline phosphatase, glucose-6-phosphate-dipotassium salt, nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), equine muscle adenosine-5'-triphosphate-disodium salt, and ascorbic acid were from Sigma Chemical Company; ethylenediamine tetraacetic acid (EDTA) from Matheson, Coleman & Bell; myo-inositol and 2-deoxy-D-glucose from Nutritional Biochemicals; α-methylmannoside from General Biochemicals, Inc.; 3% SE-30 (w/w) on Supelcoport 80/100 mesh from Supelco, Inc.; trimethylchlorosilane and N,N'-bis-(trimethylsilyl)-acetamide (BSA) from Pierce Chemical Company; and 5-thio-D-glucose from Pfanstiehl Laboratories.

Synthesis of 5-Thio-D-Glucose-6-Phosphate. A sample of 100 mg of 5-thio-D-glucose was dissolved in 25 ml of 150 mM Tris-HCl, pH 8.0, containing 0.9 mmoles ATP, 0.300 mmoles $MgCl_2$, and 140 units of hexokinase (1 unit producing 1 umole of D-glucose-6-phosphate from D-glucose per minute at 25° C, pH 8.5). The reaction proceeded for 24 hours at 37° C. Yield was approximately 40% based on the disappearance of 5-thio-D-glucose as determined by gas-liquid chromatography (19).

Isolation and Characterization of 5-Thio-D-Glucose-6-Phosphate. The entire reaction mixture was chromatographed on Whatman 3 MM paper in ethanol: 1 M ammonium acetate, pH 7.5, 7:3 overnight, dried and a second pass of the solvent was made for 10 hours. A test strip was cut and migration position was identified using silver nitrate reagent (20). The hexose phosphate region was eluted from the paper overnight with distilled water, evaporated, and resuspended in 10 ml of water. The sample was treated with Dowex--50 (H^+), brought to pH 7.0 with 0.5 N NaOH, dried and dissolved in 2.0 ml of distilled water. The material was passed through a 20 x 0.7 cm column of Dowex--1 x 8, 50-100 mesh, in the formate form. Although the hexose phosphate did not bind, an orange contaminant was removed and the residue after drying appeared white. This residue was resuspended in 5 ml of distilled water and stored at -80° C.

A sample of the hexose phosphate in 50 mM Tris-HCl, pH 8.0, was treated with E. coli alkaline phosphatase at 37° C for two hours. The resulting product was analyzed before and after treatment for free phosphate employing a modified method of Ames (21) and the amount of carbohydrate by the phenol-sulfuric acid assay (22). The 6-phosphate was also quantified using glucose-6-phosphate dehydrogenase as described below.

Measurement of V_{max} for Hexokinase and D-Glucose-6-Phosphate Dehydrogenase Using 5-Thio-D-Glucose or its 6-Phosphate as Substrate. For the hexokinase reaction, a final assay volume of 0.3 ml containing 0.84 mM ATP, 25 mM Tris-HCl, pH 8.0, 5.0 mM $MgCl_2$, and 0.42 mM $NADP^+$ and hexokinase, 0.025 units/assay, 1 unit equalling 1 umole of glucose-6-phosphate produced per minute at 25° C, pH 8.5 coupled with glucose-6-phosphate dehydrogenase, 4.5 units (1 unit equalling 1 umole of

6-phosphogluconate formed per minute at 25° C, pH 7.4, in the presence of $NADP^+$).

For the glucose-6-phosphate dehydrogenase reaction, a final assay volume of 0.4 ml containing 120 mM Tris-HCl, pH 8.0; 10.4 mM $MgCl_2$, and 0.9 mM $NADP^+$, pH 8.0, and 0.05 units where 1 unit equals 1 umole of 6-phosphogluconate produced per minute at 25° C, pH 7.4, in the presence of $NADP^+$.

Preparation and Assay of D-Glucose-6-Phosphate: L-myo-Inositol-1-Phosphate Synthase. Both the enzyme preparation and assay followed modified methods (23) of Barnett and Corina previously reported (10) utilizing rat testes as the enzyme source.

Gas-Liquid Chromatography. Plasma and tissues were deproteinized according to Somogyi (24) and were analyzed for free myo-inositol, 5-thio-D-glucose and 2-deoxy-D-glucose by gas-liquid chromatography according to Wells, et al. (19) using α-methyl mannoside as an internal standard. Determinations of lipid-bound myo-inositol were made on the neutral Folch extract (25) of liver or testes according to Wells, et al. (26). Quantification of the 6-phosphates of 5-thio-D-glucose and 2-deoxy-D-glucose was estimated by difference utilizing the gas chromatographic technique before and after treatment of samples with alkaline phosphatase. Samples were incubated both with and without E. coli alkaline phosphatase at 37° C, pH 8.5, deionized with the mixed bed resin, MB-3 (Rohm and Haas; Philadelphia, Pennsylvania) and the resulting levels of 5-thio-D-glucose or 2-deoxy-D-glucose determined by gas-liquid chromatography as described above. The 6-phosphate of 5-thio-D-glucose was qualitatively examined for purity and identification by gas chromatography using the method of Wells et al. (19).

Sperm Counts. Sperm counts were made using the technique of Kirton, et al. (27).

Animals. Young male (Balb C) mice weighing approximately 30 g were used in all experiments and were maintained on a stock mouse diet (Wayne Mouse Breeder Blox, Allied Mills, Inc.; Chicago, Illinois) and water ad libitum. Injections of the test compounds were made once daily administering between 0.1 and 0.2 ml of fluid per injection. Dosages for sugars and times of tissue sampling after the last injection were as indicated for each experiment in the results section. Tissues were removed, placed on crushed ice or quickly frozen in liquid Nitrogen and stored at -80° C. Blood samples were removed by cardiac puncture or in the case of the plasma glucose study (Figure 3) by the orbital bleeding technique (28) centrifuged and the plasma stored at -80° C until analyzed.

Quantification of Glucose-6-Phosphate and Adenosine-5'-Triphosphate. Glucose-6-phosphate (G-6-P) and adenosine-5'-triphosphate were determined after extraction with perchloric acid and spectrophotometric determination utilizing the method of Lowry et al. (29).

RESULTS

Synthesis of 5-Thio-D-Glucose-6-Phosphate. Hexokinase from yeast was used to catalyze the synthesis of 5-thio-D-glucose-6-phosphate. Previous workers (13, 29) have noted that 5-thio-D-glucose is a poor substrate for phosphorylation by hexokinase. Our investigations have also found this to be true (see Table 1); however, if adequate levels of enzyme and sufficient time (24 hours) were employed, we obtained a conversion of 40 $\pm$ 2% (mean $\pm$ S.D.) of the 5-thio-D-glucose to its 6-phosphate in three separate preparations.

TABLE 1

A Comparison of V_{max} for D-Glucose and 5-Thio-D-Glucose and Their 6-Phosphate Derivatives in Two Enzyme Systems

SUBSTRATE	ENZYME	V_{max} ($\frac{nmoles}{min}$)[a]	FOLD DIFFERENCE
D-Glucose	Hexokinase	175.0 ± 1.4	206
5-Thio-D-Glucose		0.85 ± 0.04	
D-Glucose-6-Phosphate	Glucose-6-Phosphate Dehydrogenase	157.3 ± 1.7	22.5
5-Thio-D-Glucose-6-Phosphate		7.00 ± 0.08	

[a]Each V_{max} is the mean ± S.D. for three separate determinations.

TABLE 2

Quantification of 5-Thio-D-Glucose-6-Phosphate

Alkaline Phosphatase Treatment		Analytical Methods[a]				
		G6PDH	HK-G6PDH	Phenol-H^+	GLC	Inorg P
YIELD (umoles)	-	200	---	194	---	0
	+	0	196	198	193	197

[a]All methods were as indicated in the Methods section. Yield has been converted to total umoles recovered from an isolation procedure and is typical of other preparations.

Isolation, Identification and Quantification of 5-Thio-D-Glucose-6-Phosphate. Isolation of the product by paper chromatography produced a product with the following properties. Conversion of the 6-phosphate to the hydrogen form by Dowex--50 released a colored contaminant which was satisfactorily removed by the Dowex--1 (formate) column.

Table 2 presents the quantitative analysis of products of 5-thio-D-glucose-6-phosphate before and after treatment with alkaline phosphatase. The results indicated no significant contamination of the preparation by 5-thio-D-glucose or inorganic phosphate.

Figure 1 shows gas chromatographic analysis of A) glucose and 5-thio-D-glucose and B) the 6-phosphates of glucose and 5-thio-D-glucose. Gas chromatography of commercial preparations of 5-thio-D-glucose revealed a single peak (peak 3, 8.2 minutes) which was retained longer than either α (peak 1, 4.5 minutes) or β-D-glucopyranose (peak 2, 6.8 minutes) on 3% SE-30 (w/w) on Supelcoport (80/100 mesh). Gas chromatographic analysis of the purified yeast hexokinase enzymatic reaction product showed a similar pattern with a single 5-thio-D-glucopyranose-6-phosphate peak (peak 3, 12.9 minutes) eluting more slowly than α (peak 1, 8.5 minutes) and β (peak 2, 9.3 minutes) -D-glucopyranose-6-phosphates. Conditions for the chromatography of the phosphorylated compounds are given in Figure 1.

Inhibition of D-Glucose-6-Phosphate: L-myo-Inositol-1-Phosphate Synthase (Synthase) by 5-Thio-D-Glucose-6-Phosphate (5TG-6-P). Rat testes was chosen as the source for the synthase because of the high activity of the enzyme present in that tissue (8). The average specific activity of the preparation was found to be 0.40 umoles

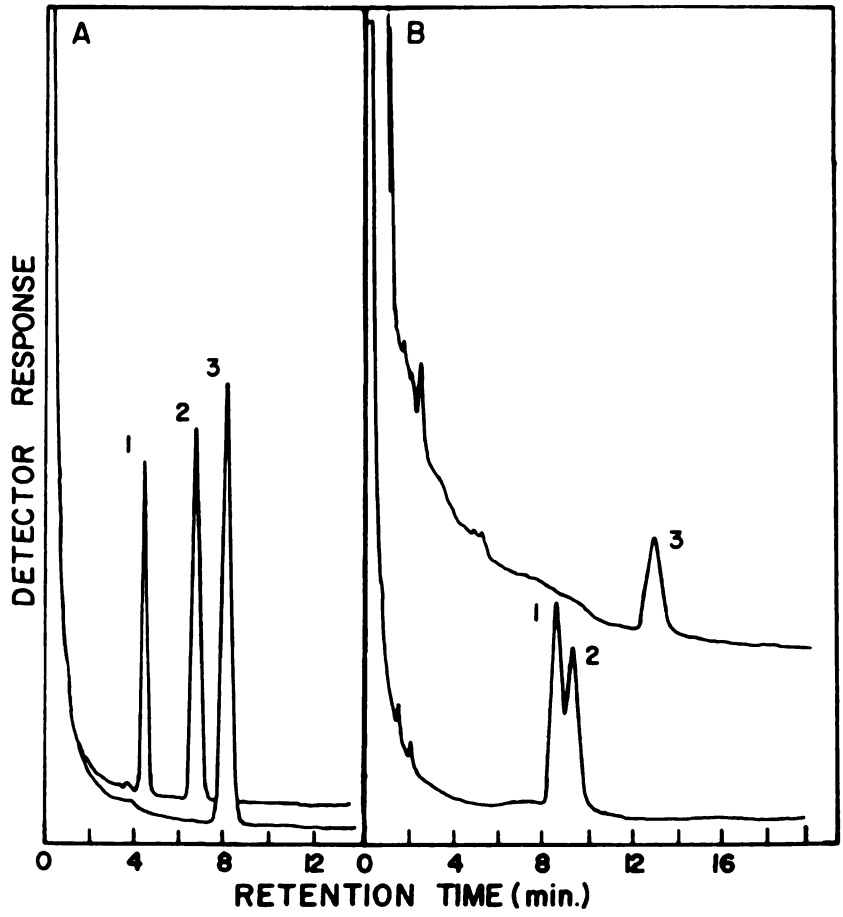

Figure 1. Gas Chromatographic Analysis of A) D-Glucose and 5-Thio-D-Glucose and B) D-Glucose-6-Phosphate and 5-Thio-D-Glucose-6-Phosphate. All samples were analyzed on a 1.8m by 3mm glass column of 3% SE-30 on Gas Chrome Q (100-120 mesh) in a Hewlett-Packard Model 302 gas-liquid chromatograph using N_2 as the carrier gas and a flame ionization detector. In A) the running temperature was 190° C. In B) the running temperature was 210° C.

L-myo-inositol-1-phosphate synthesized/hour/mg protein. In the absence of inhibitor (5TG-6-P), the apparent K_m for D-glucose-6-phosphate (G-6-P) was 0.51 mM. Four levels of inhibitor ranging from 0.10 mM to 1.00 mM were tested and the results are shown in a Lineweaver-Burk plot (30) (Figure 2A). Graphic analysis showed competitive inhibition, both by Lineweaver-Burk (Figure 2A) and Dixon plots (31) (Figure 2B). The K_i for 5-thio-D-glucose-6-phosphate, as determined from the Dixon plot was 0.33 mM which is similar to the K_m for D-glucose-6-phosphate. An additional determination on separate preparations of both enzyme and 5TG-6-P yielded equivalent results for both the K_m of G-6-P and the K_i of 5TG-6-P. Evaluation of the data was accomplished using a computer program employing a least squares best fit procedure (32).

V_{max} for 5-Thio-D-Glucose with Hexokinase and its 6-Phosphate with Glucose-6-Phosphate Dehydrogenase. Comparison of the V_{max} for D-glucose and 5-thio-D-glucose with hexokinase revealed a 206-fold difference (Table 1) indicating that 5-thio-D-glucose is a relatively poor substrate for hexokinase. Similar V_{max} comparisons for the respective 6-phosphates with D-glucose-6-phosphate dehydrogenase showed that the thio sugar phosphate is not as good a substrate as D-glucose-6-phosphate, but the difference is an order of magnitude less (22.5-fold) than that observed for the D-glucose-6-phosphate.

Effects of the Administration of 5-Thio-D-Glucose, 2-Deoxy-D-Glucose and D-Glucose on Plasma Glucose Levels. Figure 3 shows the effects of intraperitoneal administration of 5-thio-D-glucose (5TG), 2-deoxy-D-glucose (2DG), D-glucose (G) and saline (S) on plasma glucose in mice. All sugars were administered at doses of 50 mg/kg

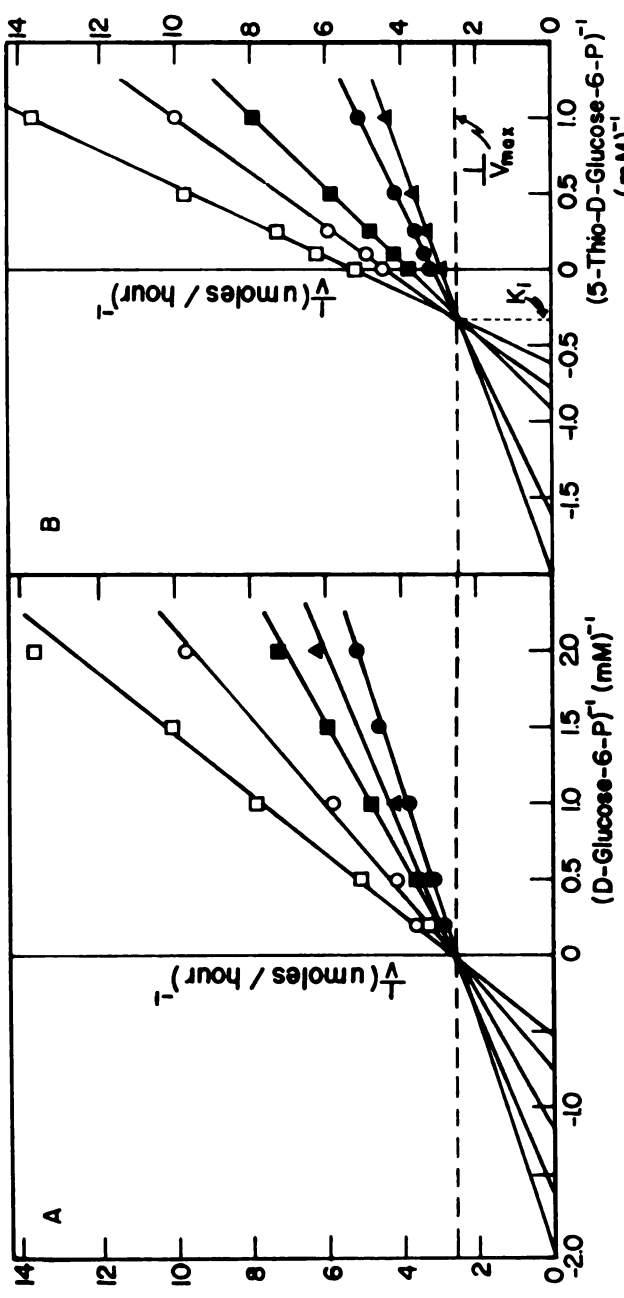

Figure 2. A) Lineweaver-Burk and B) Dixon Graphic Analysis of the Inhibition by 5-Thio-D-Glucose-6-Phosphate of the Synthesis of L-myo-Inositol-1-Phosphate from D-Glucose-6-Phosphate. In A) the following represent the concentrations of 5-thio-D-glucose-6-phosphate employed: ●, 0 mM; ▲, 0.10 mM; ■, 0.25 mM; ○, 0.50 mM; □, 1.00 mM. In B) the following represent the concentrations of D-glucose-6-phosphate employed: □, 0.50 mM; ○, 0.67 mM; ■, 1.00 mM; ●, 2.00 mM; ▲, 5.00 mM. $(V_{max})^{-1}$ and in B) $(K_i)^{-1}$ have been indicated in the figures.

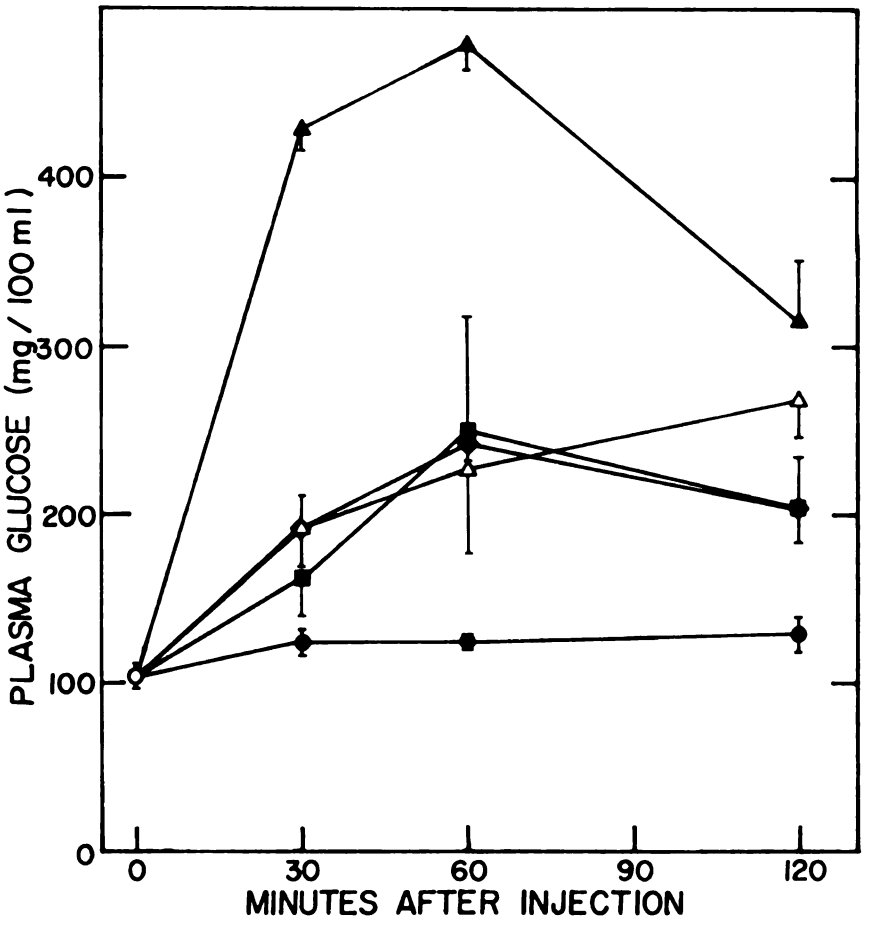

Figure 3. Effects of the Administration of a Single Dose of 5-Thio-D-Glucose, 2-Deoxy-D-Glucose, D-Glucose and Saline on Plasma Glucose Levels in Mice. The following represent the sugars or saline and their respective doses: ●, saline; ■, D-glucose; ◆, 2-deoxy-D-glucose; and △, 5-thio-D-glucose all at 50 mg/ kg body weight; and ▲, 5-thio-D-glucose at 250 mg/ kg body weight. Dosage volumes were between 0.1 and 0.2 ml. Each value represents the mean ± S.D. for 3 mice.

body weight, the volume of administration being kept less than 0.2 ml. Saline was administered as a 0.9% solution and an additional group of mice was given 5TG at 250 mg/kg body weight (5TG-5). Hyperglycemia was observed to reach its peak value for glucose and 2DG groups from one to two hours after administration. 5-Thio-D-glucose showed a rapid decline after one hour in the high dosage group (5TG-5) while in the low dosage group (5TG-1, 50 mg/kg body weight) blood glucose was continuing to rise at two hours.

In Vivo Experimentation. 2-Deoxy-D-glucose-6-phosphate (10) and, in this report, 5-thio-D-glucose-6-phosphate have been shown to be competitive inhibitors of L-myo-inositol-1-phosphate synthase. Therefore, experiments were designed to determine the effects of 5-thio-D-glucose (5TG) and 2-deoxy-D-glucose (2DG) administration might have on in vivo myo-inositol levels.

Levels of glucose or glucose analog injected were all 50 mg/kg body weight/day for seven days, and tissue was taken 24 hours after the final injection (Experiment I, Table 3). Plasma levels of myo-inositol appeared unchanged, although wider variations as compared with controls were noted in the 2DG and 5TG injected animals. Testicular tissue in response to 2DG, showed significantly ($p<0.005$) reduced levels of free myo-inositol (34.5% of control). 5-Thio-D-glucose did not elicit a change in plasma myo-inositol levels at the dose given. However, in this experiment, sampling of tissue 24 hours after the last injection did not account for potential transient effects of 5TG administration on tissue or plasma myo-inositol levels. In Experiment II (Table 4), levels of glucose and 2DG injected were identical to those in Experiment I, and 5TG administration was increased to five times that in

TABLE 3

Effects of 5-Thio-D-Glucose and 2-Deoxy-D-Glucose Administration on myo-Inositol Levels of Mouse Plasma and Testes - Experiment I

ANIMALS[a]	TISSUES[a]		
	n	Plasma	Testis
Control	3	20.6 ± 1.8	2.900 ± 0.118
5 TG	4	23.7 ± 3.9[c]	2.757 ± 0.171[c]
2 DG	3	23.4 ± 5.7[c]	1.000 ± 0.029[b]

[a]Values represent the mean ± S.D. for n animals, plasma values are in uM and tissue levels are in umoles/ g of wet weight tissue. Animals were injected once daily intraperitoneally with 50 mg/ kg body weight of glucose (control), 5-thio-D-glucose (5TG) or 2-deoxy-D-glucose (2DG) for 7 days. Tissue sampling was performed 24 hours after the last injection.
[b]$p<0.005$
[c]not significant

TABLE 4

Effects of 5-Thio-D-Glucose and 2-Deoxy-D-Glucose Administration on myo-Inositol Levels of Mouse Plasma and Tissues - Experiment II

Animals	Days Inj. (n)	TISSUES[a]			
		Plasma	Testis	Brain	Liver
Control	3 (5)	31.6 ± 5.0	2.90 ± 0.31	4.13 ± 0.20	0.295 ± 0.065
	7 (5)	27.6 ± 7.8	3.09 ± 0.57	4.03 ± 0.28	0.235 ± 0.027
5 TG	3 (5)	31.3 ± 1.5[e]	4.16 ± 0.32[c]	4.05 ± 0.27[e]	0.275 ± 0.031[e]
	7 (6)	32.2 ± 4.7[e]	4.10 ± 0.38[d]	4.10 ± 0.36[e]	0.337 ± 0.056[d]
2 DG	3 (5)	33.1 ± 8.2[e]	1.84 ± 0.36[c]	3.92 ± 0.66[e]	0.227 ± 0.034[e]
	7 (6)	14.3 ± 3.6[d]	1.03 ± 0.17[b]	3.90 ± 0.53[e]	0.185 ± 0.022[d]

[a]Values represent the mean ± S.D. of n animals, plasma values of myo-inositol are in uM and tissue values are in umoles/ g of wet weight tissue. Animals were injected once daily intraperitoneally with 50 mg/ kg body weight of glucose (control) or 2-deoxy-D-glucose (2DG) or with 250 mg/ kg body weight of 5-thio-D-glucose (5TG) for the indicated time period. Statistics: b. $p<0.001$; c. $p<0.005$; d. $p<0.02$; and e. not significant at the 5% level.

the first experiment. Tissues examined were the same with the addition of brain and liver. Animals were sacrificed after three and seven days of treatment and tissue was sampled three to four hours after the last injection. Table 4 shows the results obtained for the determination of free myo-inositol in selected tissues. As in the first experiment, plasma variations were negligible except after seven days for 2DG treated animals. In 2DG treated animals, the seven day plasma level of myo-inositol was 51.8% of the control value. Brain free myo-inositol levels were unaffected by 2DG or 5TG administration. No significant differences were observed as measured by the Student's two-tailed t test.

Liver showed no significant changes in free myo-inositol after three days of treatment with 5TG or with 2DG. After seven days, however, significant elevation (143% of control) of myo-inositol in liver was evident for the 5TG treated animals ($p<0.02$). Concomitantly, 2DG caused a significant decrease in liver free myo-inositol (78.6% of control, $p<0.02$).

Although significant deviations from the control were observed for liver and plasma samples, the largest difference occurred in the testes of the experimental animals. In animals treated with 2DG, the decrease in testicular free myo-inositol followed a time course that was nearly linear for seven days. Final values at seven days duplicated those in Experiment I at a level of 33.3% of the control value ($p<0.001$). Animals treated with 5TG showed a significant elevation in testicular free myo-inositol levels at both three ($p<0.005$) and seven ($p<0.02$) days of administration when compared with respective controls.

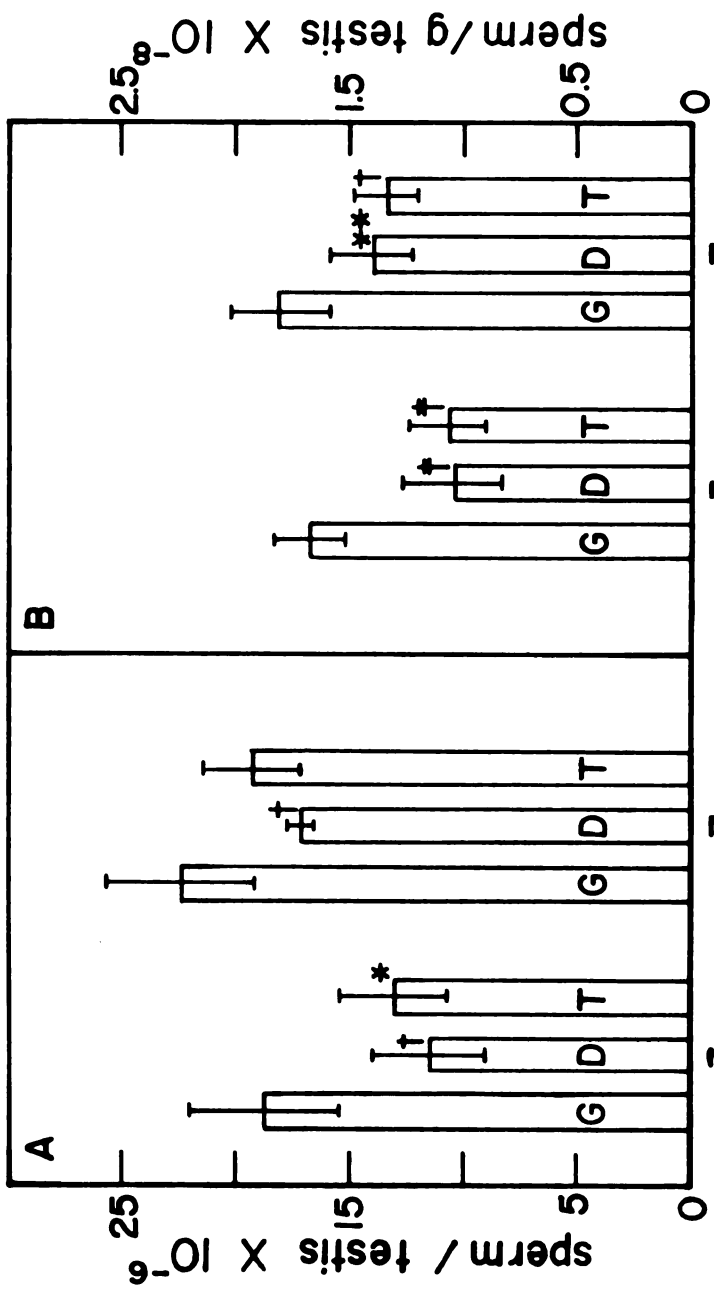

Figure 4. Effects of D-Glucose, 2-Deoxy-D-Glucose, and 5-Thio-D-Glucose Administration on Sperm Count in Mice. Each value represents the mean ± S.D. for 5 mice with the exception of 7 day deoxyglucose and thioglucose values for which n=6. G represents D-glucose treated animals, D represents 2-deoxy-D-glucose treated animals and T represents 5-thio-D-glucose treated animals. In A) values are expressed as number of sperm/ testis while in B) they are expressed as number of sperm/ g testis. Significance is indicated as follows: * , $p<0.05$; **, $p<0.025$; †, $p<0.02$; ‡, $p<0.01$.

In order to correlate possible effects of myo-inositol on fertility, sperm counts were taken for the treated and control mice. Figure 4 shows the results of the sperm counts. Data are listed as both number of sperm per testis and number of sperm per gram testis. A significant decrease in number of sperm/g testis was observed for both the 5TG (3 days, $p<0.01$ and 7 days, $p<0.02$) and 2DG animals (3 days, $p<0.01$ and 7 days, $p<0.025$) when compared with controls.

Experiment III was designed to examine the effects of short term administration of 2DG and 5TG on plasma and testicular myo-inositol and plasma glucose. Table 5 shows the effects of a single injection of the selected sugars and saline on plasma glucose and myo-inositol, two hours after the injection. Saline injected animals showed an elevation in blood glucose presumably caused by ether administration to remove tissue samples. Hyperglycemic responses to the glucose analogs reflected those observed in Figure 3. Plasma myo-inositol levels revealed significant changes ($p<0.05$) for mice given 250 mg/kg body weight 5TG. After two hours only trace amounts of 5TG remained in the plasma as determined by gas chromatography. Testicular myo-inositol levels (see Table 8) revealed different effects from those observed in the other experiments upon short term administration of 2DG and 5TG. Though not significant, elevations in testicular myo-inositol were noted for 2DG and both 5TG dosages but not for glucose.

Experiment IV examined long term effects of the administration of 5-thio-D-glucose, 2-deoxy-D-glucose and glucose on myo-inositol in testes, liver and plasma and on energy metabolites (G-6-P, ATP) in testis and liver in mice. Administration of the sugars was performed once a day for seven days. Tissue samples were removed one hour after the last injection and were quickly frozen in liquid nitrogen. 2DG

TABLE 5

Effects of the Administration of 5-Thio-D-Glucose, 2-Deoxy-D-Glucose, and D-Glucose in Mice on Plasma Glucose and myo-Inositol Levels

Group	Experiment #[b]	Plasma[a]		
		Glucose	2DG or 5TG	myo-Inositol
S	III	199.1 ± 23.6	---	40.2 ± 7.0
	IV	199.5 ± 13.9	---	68.5 ± 9.1
G	III	235.2 ± 28.7	---	55.9 ± 9.6
	IV	213.2 ± 11.3	---	57.1 ± 7.3
2DG	III	235.4 ± 39.2	ND	55.1 ± 8.3
	IV	216.2 ± 11.7	ND	33.0 ± 2.8
5TG-1	III	298.8 ± 20.6	1.68 ± 0.44	46.9 ± 7.8
	IV	266.8 ± 57.3	1.38 ± 0.35	54.9 ± 7.6
5TG-5	III	316.1 ± 40.9	2.32 ± 0.52	63.2 ± 9.2[c]
	IV	453.6 ± 36.0	3.83 ± 0.74	38.6 ± 13.8[c]

[a] Values represent the mean ± S.D. for 5 animals. D-Glucose, 2-deoxy-D-glucose and 5-thio-D-glucose values are in mg/dl and myo-inositol levels are expressed as uM. ND means non detected.

[b] For Experiment III, animals were injected once with 50 mg/kg body weight of glucose (G), 2-deoxy-D-glucose (2DG) and 5-thio-D-glucose (5TG-1) or saline (0.9%) in a volume of not more than o.2 ml. Another group received 250 mg/kg body weight of 5-thio-D-glucose (5TG-5). Tissues were removed two hours post injection. For Experiment IV, animals received dosages as in Experiment III once daily for 7 days. Tissue was removed and quick frozen in liquid N_2 one hour after the last injection.

[c] Significance = $p<0.05$

animals showed a significant reduction ($p<0.02$) in plasma myo-inositol levels similar to that observed in Experiment II (Table 4). Glucose and low dose 5TG showed no significant effects on plasma myo-inositol levels. Animals given the high dose of 5TG, however, revealed a significant reduction ($p<0.02$) of plasma myo-inositol which may be attributed to hyperglycemia and possible glucosuria and inositoluria.

Glucose administration produced no effects on the levels of either free or lipid-bound myo-inositol in the testis or liver of mice when compared with saline injected animals (Table 6). Significant reductions ($p<0.001$) in free myo-inositol were observed in testis and liver upon administration of 2DG. Levels of lipid-bound myo-inositol, however, remained unchanged in either tissue. At both doses given, 5TG produced slight but insignificant mean elevations in free myo-inositol in both liver and testes. Lipid-bound myo-inositol levels showed insignificant mean increases in the testes of high 5TG dosed animals.

The tissue metabolites (Table 7) D-glucose-6-phosphate (G-6-P) and adenosine 5'-triphosphate (ATP) were examined in order to correlate the level of the precursor (G-6-P) of myo-inositol synthesis in response to administration of glucose, 2-deoxy-D-glucose and 5-thio-D-glucose. Table 7 shows the data for the analysis of testes and liver tissue for G-6-P and ATP. In testes, while ATP levels did not vary significantly, G-6-P levels showed insignificant mean elevations above the saline controls for all the sugars and dosages examined. Significant changes ($p<0.01$) in G-6-P levels, however, were observed only after injection of 250 mg/kg of body weight 5TG. For liver G-6-P, although levels appeared to be reduced for 5TG administered animals no significance could be attached to those observations. Liver ATP levels

TABLE 6

Effects of 5-Thio-D-Glucose, 2-Deoxy-D-Glucose and D-Glucose Administration on Free and Lipid-Bound myo-Inositol Levels of Mouse Testis and Liver - IV.

GROUPS	myo-Inositol Content[a]			
	Testis		Liver	
	Free	Lipid-Bound	Free	Lipid-Bound
S	2.369 ± 0.076	0.584 ± 0.057	0.221 ± 0.028	2.458 ± 0.089
G	2.339 ± 0.082	0.593 ± 0.053	0.229 ± 0.019	2.586 ± 0.117
2DG	0.906 ± 0.120	0.582 ± 0.067	0.140 ± 0.015	2.449 ± 0.161
5TG-1	2.687 ± 0.194	0.583 ± 0.066	0.261 ± 0.054	2.636 ± 0.153
5TG-5	2.635 ± 0.325	0.795 ± 0.144	0.270 ± 0.081	2.509 ± 0.289

[a]Values represent the mean ± S.D. for 5 animals. Values are expressed as umoles of myo-Inositol/g wet weight tissue. See Table 5 for procedures and methods in Experiment IV.

TABLE 7

Effects of 5-Thio-D-Glucose, 2-Deoxy-D-Glucose and D-Glucose Administration on D-Glucose-6-Phosphate and Adenosine-5'-Triphosphate Levels of Mouse Testes and Liver - Experiment IV.

GROUPS[b]	Tissues[a,c]			
	Testis		Liver	
	G-6-P	ATP	G-6-P	ATP
S	43.7 ± 10.9	2.03 ± 0.18	348 ± 35	1.97 ± 0.22
G	62.3 ± 7.9	1.89 ± 0.14	379 ± 76	1.86 ± 0.44
2DG	55.0 ± 11.1	2.28 ± 0.29	362 ± 66	1.74 ± 0.48
5TG-1	53.2 ± 16.0	2.16 ± 0.21	309 ± 52	1.79 ± 0.31
5TG-5	69.9 ± 6.1	2.18 ± 0.17	302 ± 35	2.08 ± 0.11

[a]Values are the mean ± S.D. for 5 animals and are expressed as nmoles/g wet weight tissue (G-6-P) or umoles/g wet weight tissue (ATP).
[b]Groups are defined and treated as in Table 5.
[c]Values were determined as indicated in the Methods section.

TABLE 8

Effects of 5-Thio-D-Glucose and 2-Deoxy-D-Glucose Administration on Testes myo-Inositol Content with Time

	Testicular myo-Inositol[A]				
Exp. #	I	II	II	III	IV
Days of Inj.	7	7	3	1	7
Sample[c] post Inj.	24 hr.	3-4 hr.	3-4 hr.	2 hr.	1 hr.
GROUP[b]					
S	-----	-----	-----	2.64 ± 0.09	2.37 ± 0.08
G	2.90 ± 0.12	3.10 ± 0.57	2.90 ± 0.31	2.63 ± 0.25	2.34 ± 0.08
2DG	1.00 ± 0.03	1.03 ± 0.17	1.85 ± 0.36	3.10 ± 0.35	0.91 ± 0.10
5TG-1	2.76 ± 0.17	-----	-----	2.98 ± 0.18	2.69 ± 0.30
5TG-5	-----	4.10 ± 0.38	4.16 ± 0.32	2.90 ± 0.18	2.64 ± 0.30

[a]Each value is the mean ± S.D. for five animals expressed as umoles of myo-inositol/ g wet weight tissue. Specific experimental treatments are discussed in detail in the Results section.

[b]All groups were treated as described in the text.

[c]Sample post Inj. represents the time after the last injection at which the tissue was sampled.

did not change during any of the treatments. The 6-phosphate of 5-thio-D-glucose and 2-deoxy-D-glucose were not detected in the tissues obtained in this study.

Table 8 shows a summary of the testicular free myo-inositol levels for the experiments reported herein. Short term effects of high dosages of 5TG and longer term effects of 2DG on myo-inositol in testes were observed.

DISCUSSION

myo-Inositol has been demonstrated to be required for growth and survival for a variety of organisms including yeast, fungi, mammals and both normal and cancerous human and murine cell lines (15, 17, 33, 34). In addition, biosynthesis of myo-inositol from D-glucose-6-phosphate has been demonstrated in most of these organisms or their tissues *in vitro* (35) and *in vivo* (36 - 38). Therefore, to more fully investigate the role of tissue myo-inositol and effects of myo-inositol deficiency it would be necessary to remove exogenous sources and inhibit biosynthesis. Most of the effort in searching for the inhibitor of myo-inositol synthesis has been directed at the synthase reaction because of its rate limiting role (1) and the importance of the substrate, D-glucose-6-phosphate in other cellular reactions.

The substitution of sulfur for oxygen in 5-thio-D-glucose-6-phosphate (5TG-6-P) suggested potential interference in the cyclization reaction of the synthase since carbon number 5 engages in an intramolecular oxidation-reduction sequence (5). Enzymatic synthesis via hexokinase and isolation of 5TG-6-P produced a product with no detectable contamination as judged by carbohydrate, phosphate and gas-liquid chromatographic analysis. The 6-phosphate of

5-thio-D-glucose proved to be a poor substrate in the glucose-6-phosphate dehydrogenase reaction (Table 1). Values similar to those in Table 1 were obtained for the V_{max} of the hexokinase catalyzed reaction by other investigators (13), but to our knowledge, no previous comparisons of 5-thio-D-glucose-6-phosphate and glucose-6-phosphate have been made for the dehydrogenase catalyzed reaction.

5-Thio-D-glucopyranose synthesized by Feather and Whistler (11) was reported to have an optical rotation of α (39). A commercial preparation from Pfanstiehl Laboratories also shows a single peak by gas chromatographic analysis, presumably corresponding to the α-anomer (see Figure 1A). Configuration appears to be retained upon the enzymatic conversion of the thio sugar to its 6-phosphate via yeast hexokinase since only a single peak is observed by gas chromatography but this is by no means established (Figure 1B). This would suggest the ability of yeast hexokinase to utilize a single anomeric form (probably α) during the enzymatic reaction since the thio sugar reportedly does not mutarotate in water (39, Burton and Wells, unpublished results). Similarly, yeast glucose-6-phosphate dehydrogenase must also be able to react with a single anomeric form since we have not observed the mutarotation of the 5-thio-D-glucose-6-phosphate in water by gas chromatography, unless both peaks are superimposed. This, however, is not the case where α and β-D-glucopryanose-6-phosphate are similarly treated. Since the singular form of the 6-phosphate is probably α, this would also suggest that α is a form of D-glucose-6-phosphate, which can function at the active site of L-myo-inositol-1-phosphate synthase (EC 5.5.1.4).

2-Deoxy-D-glucose-6-phosphate (2DG-6-P) was demonstrated to be a

strong competitive inhibitor (K_i = 20 uM) of the synthase reaction (10). Similarly, 5-thio-D-glucose-6-phosphate (5TG-6-P) was found to be a competitive inhibitor. The similarity of the K_m (G-6-P, 0.5. mM) and K_i (5TG-6-P, 0.33 mM) suggest that both G-6-P and 5TG-6-P bind the active site of synthase with comparable affinity. Similar affinity for the active site may imply no important role for the heteroatom in the binding of the hexose phosphate at the active site, not unlike similar observations made by Barnett et al. (40) with regard to the importance of the ring oxygen in sugar transport in the intestine. This does support, however, the importance of the heteroatom and bonds to that atom in the mechanism of the cycloaldolization since we have not been able to observe the synthesis of the sulfur analog of L-myo-inositol-1-phosphate. Further work is indicated in order to determine the usefulness of 5-thio-D-glucose-6-phosphate in the further elucidation of the synthase mechanism.

Previous studies on the biosynthesis of myo-inositol in mammals have been centered on rat testes due to the high synthetic activity presentin this tissue (8). Middleton and Setchell (41) have shown in vivo that the large majority of myo-inositol in the testes of the ram is due to synthesis from glucose. Using this fact, mice were examined for the effect the inhibitors 2-deoxy-D-glucose and 5-thio-D-glucose have on the levels of myo-inositol in testes and other selected tissues.

Hoffman and Whistler (13) have reported that intraperitoneal injections of 5TG caused hyperglycemia and that 5TG may interfere with D-glucose entry into the cell. Hellman et al. (42) found 5TG inhibited D-glucose-mediated insulin release, but in other studies when insulin

was administered concomittantly with 5TG, the hyperglycemia was not observed (13). The inhibition of spermatogenesis by 5TG observed in mice was suggested to be due to prevention of D-glucose uptake by testicular cells (15) and decreased cellular glucose might be expected to interfere with synthesis of myo-inositol. However, in the 5TG treated animals, we have observed an elevation of free myo-inositol. Sources for this increased amount of myo-inositol could include synthesis (especially in the testes (41)), degradation of phosphoinositides and alterations in cellular transport of myo-inositol. Examination of glucose-6-phosphate and ATP levels in both testis and liver showed no significant effects of 5-thio-D-glucose on these metabolites at levels shown to inhibit spermatogenesis (15). Dosages higher than 100 mg/kg body weight (250 mg/kg body weight), known to cause severe inhibition of spermatogenesis after seven days (15) caused significant elevation in glucose-6-phosphate levels in the testes. This elevation in glucose-6-phosphate coincided with the increase in mean free myo-inositol level observed in testis but conflicts with the reported reduced glucose transport as a potential cause of the infertility observed with this compound (15). Increases in lipid-bound myo-inositol were observed only for the highest level of 5TG administered and coincided well with the elevated levels in free myo-inositol (Tables 4 and 6). Free and lipid-bound myo-inositol and D-glucose-6-phosphate levels in the testis support the biosynthetic origin of the elevated myo-inositol in 5TG treated animals. Reduction of intracellular glucose by 5TG (15) is not suggested by G-6-P and ATP measurements and therefore, another mechanism may be involved (43). In two dietary studies, (44, 45) effects on the fertility of laboratory animals by myo-inositol were reported. In Experiment II, sperm counts were made in order to

correlate potential effects of the apparent deficiencies or excesses of myo-inositol on fertility. Although the three day counts are lower, both time points show a significant decrease in the sperm population when compared with controls. These preliminary results suggest that a decrease or an elevation in the normal testicular levels of myo-inositol may affect the production of sperm and the fertility of these animals.

2-Deoxy-D-glucose-6-phosphate is known to be synthesized by hexokinase, to inhibit phosphoglucoisomerase and is a poor substrate for D-glucose-6-phosphate dehydrogenase (46). In addition, inhibition of L-myo-inositol-1-phosphate synthesis (10) suggests it is a likely candidate for in vivo inhibition of myo-inositol synthesis. Decreases in tissue free myo-inositol levels in vivo for testes and liver upon administration of 2-deoxy-D-glucose (2DG) are consistent with this possibility. No evidence has been presented for the interference of glucose or glucose analogs in the cellular transport of myo-inositol (46). 2-deoxy-D-glucose inhibition of the synthesis of L-myo-inositol-1-phosphate, therefore, most likely acts via the 6-phosphate. Turnover of myo-inositol in testicular tissue reportedly occurs slowly (41), which correlates with the observed long term effects of 2-deoxy-D-glucose administration on myo-inositol levels in the testis. The time dependent decrease in testicular myo-inositol (Table 4) may be interpreted as the result of either extremely slow turnover rates accompanied by continued inhibition of synthesis or as a gradual increase in the buildup of cellular biosynthetic inhibitor (2DG-6-P). The inability to detect the 6-phosphate of 2-deoxy-D-glucose in the tissues examined remains an enigma. The experimental time selected in these studies reported herein may not have been short enough and

appropriate studies will be conducted in the future.

We were unable to detect significant changes in free myo-inositol levels in brain regardless of treatment, in agreement with results for rats fed a myo-inositol deficient diet (Burton, L.E. and Wells, W.W., unpublished observations, 1975). Thus, the control of the free myo-inositol pool may be different in the brain than that of other tissues examined.

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DISCUSSION

"There are those who ask what inositol does--of what importance is it? . . .in a biological milieu it is a dynamic material. . ." (1)

The observation that fetal blood myo-inositol is consistently ten-fold that of the respective dam was first made by Offergeld in 1906 (2) and again by Nixon in 1952 (3). Nixon (4) and Andrews et al. (5) provided further evidence that fetal biosynthesis (probably hepatic) could be the major source of this gradient. Recent work by Burton and Wells (6) has again suggested hepatic biosynthesis as the origin of fetal plasma myo-inositol. The profiles of the biosynthetic enzymes in fetal and neonatal liver and in the mammary tissue of the dam after birth correlate with myo-inositol levels in fetal plasma and milk. Dietary deprivation or supplementation of myo-inositol indicated that diet was ineffective in significantly altering fetal plasma and tissue myo-inositol levels (Chapter I). These data are accompanied by simultaneous diet and inhibitor (2-deoxy-D-glucose the 6-phosphate (7) of which is a known myo-inositol biosynthetic inhibitor) administration, presenting further evidence supporting biosynthesis as the major source.

During neonatal development reliance on dietary (mammary and milk) sources became more apparent with the decrease in hepatic biosynthetic capacity (Chapter I) and the ability of milk and dietary myo-inositol levels to influence the amount of plasma and tissue myo-inositol in the neonate (Chapter III). myo-Inositol deprivation in the neonate did not significantly affect the growth, physical development (Chapter III), or neural development (Chapter VI) of the rat and caused only a minor, transient elevation in liver lipid content between 12 and 48 days of age (Chapter III). However, intestinal flora myo-inositol production and residual milk myo-inositol content of deprived dams could not be

excluded as potential exogenous sources of myo-inositol for the neonate prior to weaning. Intestinal flora was later shown to be a significant exogenous supply of myo-inositol when dams fed a myo-inositol deficient diet without antibacterial agents failed to develop the fatty liver associated with myo-inositol deprivation (Chapter IV).

Immunity to alterations in growth or development of the neonate due to myo-inositol deprivation, however, may also stem from the observed developmental increase in the ability of rat tissues to retain myo-inositol (Chapter I). The relative importance of each of these sources to the stability of the neonate to myo-inositol deprivation has yet to be determined.

Interest in milk myo-inositol levels has been stimulated by observations of elevated levels of the polyol at various times during lactation for different species (6, 7). Burton and Wells (6) have suggested a possible relationship of milk myo-inositol and the maturity of the neonate at birth. In the rat, approximately 90% of the free myo-inositol in the milk has been shown to be of dietary origin (Chapter III) despite increases in mammary biosynthetic enzyme levels (6). In the same study (Chapter III), reductions in tissue free myo-inositol were observed in a variety of tissues in the developing rat as a result of maternal myo-inositol deprivation and the subsequent low myo-inositol levels of the milk. Sure (9, 10) has considered myo-inositol as a factor which lowers the mortality in newborn rats and Climenko and McChesney (11) believe myo-inositol to be necessary for the maintenance of normal lactation and thus responsible for Sure's observations.

In view of the low levels of myo-inositol found in commercial baby formulas and bovine milk (6) and the clinical practice of removing "problem" (i.e. premature, galactosemic and respiratory distressed) babies from breast feeding (high levels of myo-inositol), these infants may be subject to myo-inositol deprivation during critical periods of development.

Future work regarding milk myo-inositol must be concerned with its role and significance in the development of the neonate.

Recent studies of myo-inositol containing compounds in milk have discovered a disaccharide shown to be 6-O-β-D-galactopyranosyl myo-inositol (6-β-galactinol), the significance of which has not yet been determined (12 - 14). Levels of myo-inositol have been shown to directly affect the levels of 6-β-galactinol in both rat and human milk (13, Chapter III, Discussion Figure 1).

In a diet designed to contain adequate amounts of all known vitamins, choline, essential fatty acids (as an unsaturated fat) and protein as well as an antibacterial agent (phthalylsulfathiazole), myo-inositol deprivation revealed the discovery of a myo-inositol deficient lactation induced fatty liver. Previous reports (15, 16) have suggested the fatty liver syndrome of myo-inositol deprivation to be dependent upon saturated fats. However, these studies (Chapter III) and those of Andersen and Holub (17) suggest that saturated fats are not an absolute requirement. Absence of the syndrome during myo-inositol supplementation and during gestation and after involution suggests that both myo-inositol and the physiological requirements of lactation play an important role in the maintenance of the syndrome. Preliminary data (Burton, L.E. and Wells, W.W., unpublished results, 1976) for

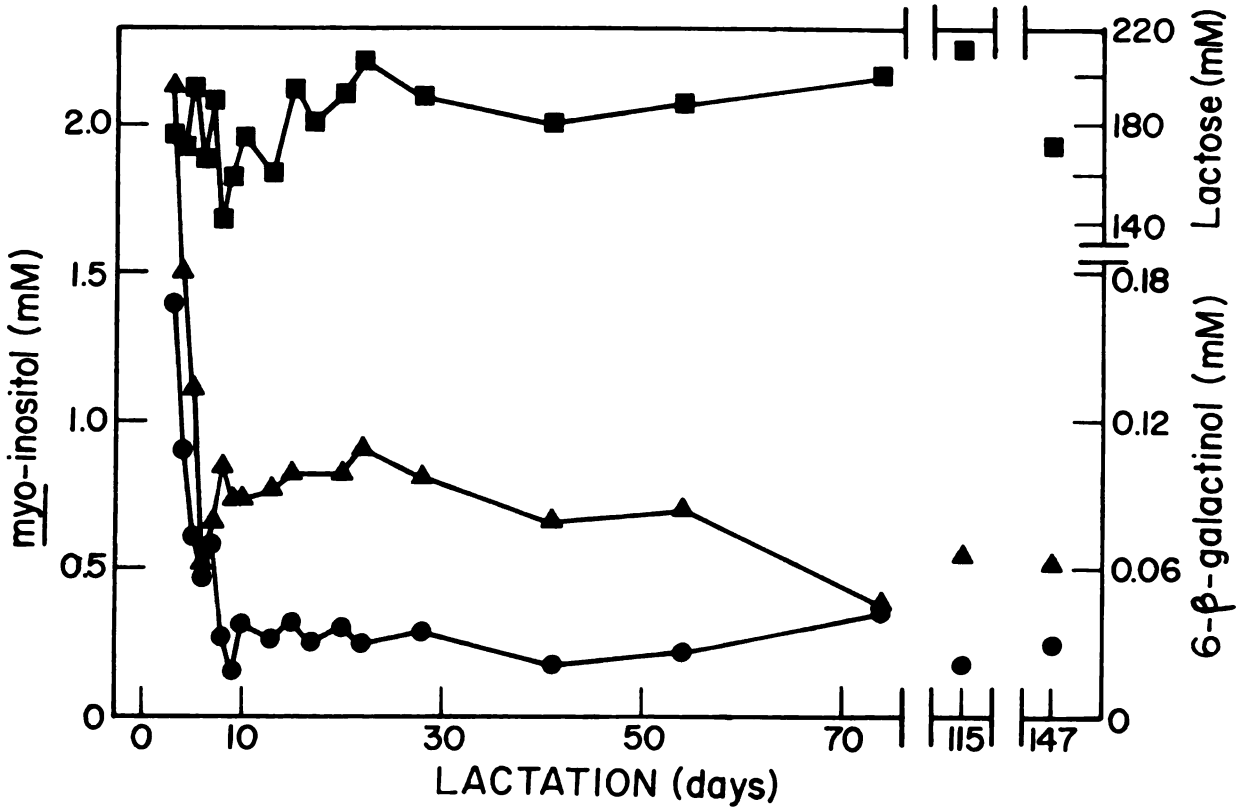

Figure 1. myo-Inositol (▲), 6-β-Galactinol (●) and Lactose (■) Levels in Human Milk. Each point represents 1 determination of the respective compound. Milk samples were all from the same subject.

example, suggests that prolonged lactation (21 - 35 days) in the rat does not significantly increase or decrease the maximum level of lipid (31% by weight) deposited during myo-inositol deprivation at 21 days of lactation. Characterization of the deposited liver lipid (Chapter IV) showed it to be 97% triglyceride and 2.5 - 3% cholesterol esters. A dual mechanism involving a block in holo-lipoprotein synthesis and secretion and increased mobilization of free fatty acids, has been suggested to explain the apparent reduction in plasma lipoprotein lipids and the concommitant elevation in plasma free fatty acid levels during lactation (Chapter IV). Reduction in liver phospholipids and especially phosphatidylinositol is the suspected source of the block in holo-lipoprotein synthesis and secretion (Chapter IV). Characteristics of the fatty liver syndrome produced by myo-inositol deprivation and lactation suggest it to be similar to that observed in choline deficiency (18) and future studies will be involved with elucidating its mechanism.

The discovery of a metabolically inert inhibitor of myo-inositol biosynthesis would greatly facilitate studies regarding the biological functions of myo-inositol. 5-Thio-D-glucose-6-phosphate has been shown to be a competetive inhibitor (K_i = 0.33 mM) of D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4) in vitro (Chapter VI). The in vivo administration of 5-thio-D-glucose in mice, however, produced elevations in testicular free myo-inositol (Chapter VI) where synthesis of myo-inositol has been shown to be high compared with other tissues (19, 20). Synthesis is suggested as the mechanism of the increase in myo-inositol as a result of the physiological effects of hyperglycemia due to 5-thio-D-glucose administration (21) producing

elevated levels of the myo-inositol biosynthetic precursor, D-glucose-6-phosphate (Chapter VI).

Inhibition of myo-inositol-1-phosphate *in vitro* has also been observed for 2-deoxy-D-glucose-6-phosphate (K_i = 20 uM) (7). Administration of 2-deoxy-D-glucose to mice produced time dependent significant reductions in testes and liver free myo-inositol without affecting levels of the biosynthetic precursor D-glucose-6-phosphate (Chapter VI). Additional evidence includes the reduction by 2-deoxy-D-glucose administration of normally high fetal plasma and tissue myo-inositol levels (Chapter I) which are believed to originate from fetal hepatic biosynthesis (6, Chapter I). Therefore, it would appear that 2-deoxy-D-glucose (in low dosages) may have merit as a potential *in vivo* inhibitor of myo-inositol synthesis.

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APPENDIX

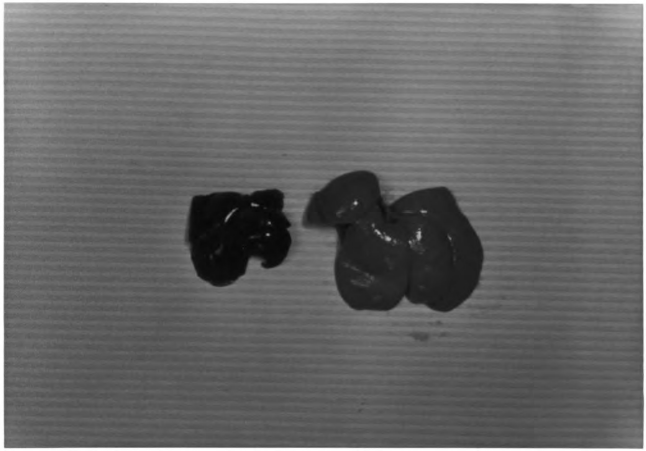

Figure 1. The Physical Appearance of the Livers of Dams Fed a myo-Inositol Supplemented or Deficient Diet after 21 Days of Lactation. Representative samples of the livers of dams fed the myo-inositol supplemented (left) and myo-inositol deficient (right) diet after 21 days of lactation are shown. The liver of the deprived dam shows the enlarged, yellowed appearance typical of other types of fatty liver. The animals sampled were of comparable total body weight.