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In Vivo Metabolite Regulation of
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IN VIVO METABOLITE REGULATION OF *myo*-INOSITOL BIOSYNTHESIS

By

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ABSTRACT

IN VIVO METABOLITE REGULATION OF
myo-INOSITOL BIOSYNTHESIS

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In vivo regulation of myo-inositol biosynthesis by the cytosolic NAD^+/NADH ratio and glucose-6-phosphate levels were investigated in mammalian systems.

Ethanol feeding of pregnant rats was utilized in an attempt to lower the fetal liver cytosolic NAD^+/NADH ratio. A significantly reduced redox state was expected to inhibit myo-inositol biosynthesis since the rate limiting enzyme, L-myo-inositol-1-phosphate synthase, has an absolute requirement for NAD^+. However, ethanol consumption by dams did not significantly alter the redox state of the fetal liver, nor were myo-inositol levels in fetal liver significantly changed.

Streptozotocin diabetes resulted in a two fold increase in testicular glucose-6-phosphate levels and a three fold increase in testicular myo-inositol. Further results suggested that in vivo testicular myo-inositol levels were primarily regulated by the size of the glucose-6-phosphate pool and that increases in myo-inositol content were not due to increases in the specific activities of the biosynthetic enzymes or myo-inositol transport. Elevated testicular glucose-6-phosphate levels were attributed to an increased flux of glucose into the testis, rather than due to increased hexokinase activity. The effects of elevated testicular myo-inositol levels on testicular sperm counts and tubulin polymerization were also examined.

DEDICATION

To my wife, Nancy, for her constant love and encouragement.

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
DMSO	Dimethylsulfoxide
EGTA	Ethylene glycol bis(β-aminoethyl ether)N, N, N', N' tetraacetic acid
EtOH	Ethanol
G6P	D-Glucose-6-phosphate
GTP	Guanosine-5'-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
K_m	Michaelis constant
NAD^+, NADH	Oxidized and reduced nicotinamide adenine dinucleotide
$NADP^+$, NADPH	Oxidized and reduced nicotinamide adenine dinucleotide phosphate
Tris	Trishydroxymethylaminomethane
V_{max}	Maximum velocity

INTRODUCTION

Objectives and Rationale

myo-Inositol has been proposed to serve several functions in nature including that of an essential growth factor for microorganisms (1-4) and mammalian cell lines (5,6), a potential vitamin in animals (7), an agent important in the maturation of spermatozoa in the mammalian male reproductive tract (8-11), and a component of phosphoinositides, important membrane constituents (12). A pathway has been described in several mammalian species for the biosynthesis of myo-inositol consisting of two enzymes, D-glucose-6-phosphate: L-myo-inositol-1-phosphate synthase (EC 5.5.1.4), an enzyme having an absolute requirement for NAD^+, and L-myo-inositol-1-phosphate phosphatase (EC 3.1.3.25) (13-18). It was the purpose of this research to investigate the in vivo regulation of the mammalian myo-inositol biosynthetic pathway by selected metabolites.

Chapter I deals with the regulation of de novo myo-inositol biosynthesis in fetal rat tissues by the cytosol NAD^+/NADH ratio. A fetal rat system was chosen since previous work in this laboratory demonstrated that fetal rat liver contained high activities of the myo-inositol biosynthetic enzymes (18). Since the rate limiting step of myo-inositol biosynthesis is at the level of the NAD^+-

linked synthase (19), a lowered cytosol $NAD^+/NADH$ ratio was expected to result in decreased myo-inositol biosynthesis. Ethanol was fed to pregnant rats in an attempt to lower the fetal cytosol $NAD^+/NADH$ ratio as a result of ethanol oxidation through the NAD^+-linked alcohol dehydrogenase of fetal rat liver. The approach used to evaluate in vivo changes in fetal myo-inositol biosynthesis was to quantitate the concentration of the free cyclitol in various fetal tissues. In order to minimize error due to myo-inositol contributions from the diet and enteric microorganisms, myo-inositol free diets and inclusion of the antibiotic phthalylsulfathiazole in the diet were utilized in some experiments. Lowered fetal myo-inositol biosynthesis in the rat fetus may be deleterious to normal growth and development.

Chapter II characterizes the in vivo regulation of myo-inositol biosynthesis in the rat testis by elevated glucose and glucose-6-phosphate levels. The rat testis was chosen since it has been shown to contain a high capacity for myo-inositol biosynthesis (13). Hyperglycemia was induced in male rats through injection of streptozotocin, a diabetogenic agent. Since the testis is not an insulin dependent tissue, hyperglycemia was expected to elevate glucose and glucose-6-phosphate levels in the testis. In response to an increase of testis glucose-6-phosphate, the substrate of the NAD^+ dependent synthase of the myo-inositol biosynthesizing pathway, increased production of myo-inositol was predicted. Elevated testicular myo-inositol levels in diabetic animals may reveal information on the role of

myo-inositol in the male reproductive tract.

Literature Survey

myo-Inositol Occurrence and Role in Growth and Development. myo-Inositol, a cyclitol first discovered by Scherer in 1850 (20), is a ubiquitous compound in nature. It exists in several forms including free myo-inositol, phosphoinositides, phytic acid and other inositol phosphate esters, 6-O-β-D-galactopyranosyl myo-inositol (6-β-galactinol); it is a constituent of glycerides, and is found as mono- and di-O-methylated derivatives (7). Phosphoinositides or myo-inositol containing phospholipids are the most predominant form of myo-inositol, serving as major components of membranes (12).

In addition to its importance in phosphoinositides, myo-inositol has been proposed to have a role in nutrition. myo-Inositol has been shown to be an essential growth factor for yeast and fungi (1-4), as well as for numerous normal and cancerous cell lines (5,6). Woolley demonstrated in 1940 that mice maintained on a myo-inositol deficient diet failed to thrive, displayed alopecia, and died within two to three weeks (21). However, conflicting results have been obtained by numerous investigators concerning the existence of an essential nutrient role for myo-inositol in mammalian systems (22-31). Absolute myo-inositol deficiency has been difficult to achieve. In vivo myo-inositol biosynthesis, degradation of phosphatidylinositol, myo-inositol production by intestinal microorganisms, and transport

of the cyclitol between tissues have all complicated efforts to obtain evidence that dietary myo-inositol is required for growth and development of mammals.

Biosynthesis of myo-Inositol. An endogenous biosynthetic pathway for myo-inositol in mammalian systems has been suspected since the original observation by Vohl (32) in 1858 that significant quantities of myo-inositol were found in the urine of diabetics. Several years later Needham (33) detected myo-inositol in the urine of rats maintained for eight months on a myo-inositol free diet. Other investigators supported the argument for endogenous biosynthesis in mammals by demonstrating that tissue levels of myo-inositol were not depleted during dietary inositol deprivation (34) even after intestinal flora suspected of supplying the cyclitol were eliminated (35).

The suggestion that myo-inositol was synthesized from glucose was originally postulated by Maquenne (36) as early as 1887 after the structural similarities between the cyclitol and the monosaccharide were realized. This hypothesis was further developed by Fischer (37) and gained support with the elucidation of the configurational similarities of glucose and myo-inositol (38). Daughaday and others (39) supplied the first direct evidence for glucose as a precursor for myo-inositol in mammals by demonstrating the incorporation of randomly labelled ^{14}C-glucose into myo-inositol. Using 6-^{14}C-glucose, Hauser (40) subsequently showed that incorporation of label into myo-inositol was faster in young

rats than in adults and that label was initially incorporated into the free myo-inositol pool and then into phospholipids. Using a rat testis homogenate system, Eisenberg, et al.(13) studied the incorporation of glucose labelled at specific carbons into myo-inositol and deduced from the labelling patterns that myo-inositol biosynthesis might occur by a direct internal aldolization of glucose. Subsequent studies in yeast (41-43), Neurospora crassa (44), rat testis (13-16), rat mammary gland (17,18), and fetal rat liver (18) resulted in the discovery of L-myo-inositol-1-phosphate synthase (EC 5.5.1.4), an enzyme which catalyzes the NAD^+ requiring internal aldolization of glucose-6-phosphate to yield L-myo-inositol-1-phosphate. myo-Inositol-1-phosphate formed in the synthase reaction is hydrolyzed specifically by L-myo-inositol-1-phosphate phosphatase (EC 3.1.3.25) to yield L-myo-inositol (43,16).

Following the discovery of these biosynthetic enzymes, investigations were conducted to elucidate the enzyme mechanisms involved. Mechanistic work concentrated on the synthase since this enzyme was determined to be rate limiting in myo-inositol biosynthesis (19). The synthase from mammalian systems was shown by a number of investigators to be a cycloaldolase of the type I class (19, 45-48). The postulated mechanism for the synthase involves an NAD^+ dependent oxidation of the C-5 carbon of glucose-6-phosphate to a keto group, followed by Schiff base formation and an aldol condensation of the C-6 and C-1 carbons to yield 2-myo-inosose-1-phosphate, and reduction to form 1-L-myo-inositol-1-phosphate. However, recent evidence presented by Sherman,

et al. argues against the proposed Schiff base intermediate in the synthase mechanism (49). It was demonstrated using testis synthase that ^{18}O was retained in myo-inositol synthesized from D-(5-^{18}O)-glucose-6-phosphate and further, no incorporation of ^{18}O into product was observed when the synthase reaction was carried out in a medium enriched in $H_2{}^{18}O$. Sherman, et al. suggested that the synthase is neither a type I or II cycloaldolase and that the cyclization process may proceed through general base catalysis. Synthase from N. crassa (50) and yeast (51) has been shown to be a type II cycloaldolase in which Zn^{2+} is the participating metal cation.

Review of Male Reproductive Tract Physiology. Spermatozoa are formed in the seminiferous tubules of the testis and are the end product of the spermatogenic function of this tissue (52). In addition to its spermatogenic function, the testis possesses an endocrine androgenic activity. Testosterone is secreted from Leydig cells, cells located in the intertubular tissue of the testis, and this hormone determines the output of seminal fluids by the accessory organs as well as secondary sex characteristics (53). Sertoli cells or "nurse cells" located in the intertubular spaces, are cells containing prominent nucleoli and nuclei. They secrete androgen binding protein in response to follicle stimulating hormone and/or testosterone (54,55). Since certain steps of spermatogenesis are dependent on testosterone (56), androgen binding protein may have an essential role

in concentrating testosterone within the seminiferous tubule fluid for binding by receptors on germ cells (54). Sertoli cells have also been demonstrated to form aggregates surrounding the apical end of the developing spermatid (57) and engage in other interactions with the developing germ cells (58). In addition to their role in spermatogenesis, Sertoli cells adjacent to each other have been shown to form tight junctions which are believed to be the basis for the blood-testis barrier (55, 57-59).

The epididymis, attached to the testis through a group of ducts (ductuli efferentes), serves as a site for the concentration, maturation, and storage of sperm. Sperm passage from the testis into the epididymis varies from a few days to several weeks, depending on the species, and depends on the peristaltic movement of the seminiferous tubules (60). There is no direct evidence that the spermatozoa move into the epididymis under their own locomotion. Sperm are concentrated within the epididymis as a result of reabsorption of as much as ninety-nine percent of the fluid produced by the testis. In this process sodium chloride is absorbed; however, glutamic acid and myo-inositol are not appreciably taken up by the epididymis(61). Phosphatidylcholine, sialic acid, and carnitine are secreted into the lumen of the epididymis. These substances are most likely incorporated into the maturing sperm as they undergo changes in membrane characteristics and increase in specific gravity and fertilizing capacity (52). Sperm remain in the epididymis in a non-motile state until ejaculation. The substrate

used by sperm for basal metabolism during this non-motile period is not known with certainty, but is thought to be endogenous phospholipid (62).

After travelling from the epididymis by way of the ductus deferens, sperm sequentially reach the seminal vesicles, prostate gland, and bulbourethral glands (Cowper's glands), known collectively as the accessory glands of the male reproductive tract. While the physiology of the accessory glands is not well understood, all are known to secrete fluids into the lumen of the sperm-carrying ducts which both dilute and facilitate the transport of sperm.

The seminal vesicle contributes substantially to the volume of the ejaculate by a thick secretion rich in fructose, the primary energy source of the sperm, and containing considerable amounts of myo-inositol (52). In addition, the vesicular secretion contains riboflavin and other flavins which give the secretion a yellowish appearance.

The secretion from the prostate is slightly acidic and contains amylase, -glucuronidase, several proteolytic enzymes and fibrinolysin, enzymes thought to be involved in the mechanism of semen coagulation (52). It is also the main source of citric acid and acid phosphatase of the semen. The secretion from the Cowper's glands is rich in sialoproteins in some species which serve to coagulate sperm after ejacultion. In rodents, coagulating glands located on the surface of the seminal vesicles are the source of a secretion which is believed to be important in the coagulation

process. In addition, in rodents, the coagulating gland secretion is a main source of fructose, together with the prostate secretion, rather than the seminal vesicles as in other mammals.

Role of myo-Inositol in the Male Reproductive Tract. Interest in the function of myo-inositol in the male reproductive tract followed the observation by Mann (63,64) that the dialyzate from boar seminal vesicle secretion contained from 40 to 70 percent myo-inositol, thus making it the richest source of the free cyclitol in nature. Semen from the boar was also found to have a high myo-inositol content (382-625 mg/dl) (65). Semen from other species has considerable concentrations of myo-inositol including human (50-60 mg/dl) (65), bull (25-46 mg/dl) (65), and ram (14-20 mg/dl) (66). Other investigators showed that myo-inositol occurs in the testis fluid of the ram at a level of over 100 times that found in the peripheral plasma (67,68). These high levels of myo-inositol in the seminal vesicle secretion, semen, and testis fluid were originally suggested to play a role in the maintenance of osmotic equilibrium in the seminal plasma, a fluid which generally contains less sodium chloride than other body fluids (64,69).

Subsequently Eisenberg and Bolden characterized an enzyme system in the testis responsible for the biosynthesis of myo-inositol (13). However, they demonstrated that the epididymis, seminal vesicle, and seminal fluid had only a slight capacity for the biosynthesis of the cyclitol. In

addition, the testis was found to store little myo-inositol, while the accessory organs, having only slight biosynthetic ability, maintained higher concentrations of the cyclitol. These authors noted that myo-inositol levels increased from testis to epididymis to seminal vesicle and based on this evidence, it was postulated that myo-inositol may play a role in the maturation of spermatozoa as they migrate from the testis through the epididymis, finally mixing with the seminal fluid at ejaculation.

The high levels of myo-inositol found in the accessory organs, in spite of low biosynthetic capabilities, suggested an ability of these tissues to concentrate inositol from the blood. Lewin and Sulimovici (70) demonstrated in rats that intraperitoneally injected radioactive myo-inositol was accumulated from the blood into the coagulating gland and seminal vesicle within two hours, and the prostate, epididymis, and ductus deferens concentrated myo-inositol at a lower rate. In addition, myo-inositol uptake by the accessory organs is thought to be under androgen control (71). In contrast to the accessory organs, myo-inositol is synthesized in the testis from glucose in preference to the utilization of preformed inositol present in the blood plasma (70,72); therefore, a testis-blood barrier for myo-inositol is thought to exist.

The site of myo-inositol synthesis within the testis was originally suggested by Eisenberg (16) to be at the level of the seminiferous tubule. Subsequent studies by Voglmayr and White (73) indicated that testicular spermatozoa have

a capacity for endogenous biosynthesis of the cyclitol and suggested that this process may occur in the cytoplasmic droplet of the testicular spermatozoan. However, the biosynthetic activity of these sperm is unlikely to entirely account for the inositol concentration observed in the testis fluid (68) since inositol levels considerably in excess of blood plasma have been observed when sperm numbers were less than 0.01% of normal (74). In contrast to testicular spermatozoa, epididymal spermatozoa are unable to synthesize inositol (16,73); the regulatory or developmental process by which inositol biosynthesis in spermatozoa is controlled has not been elucidated. In addition, the metabolic role of myo-inositol synthesized by testicular spermatozoa is not clear. The endogenous inositol may be involved in resynthesis of phosphatidylinositol which is utilized by the spermatozoa during their period of maturation in the epididymis (18), presumably after hydrolysis and oxidation of the fatty acid components (9). Another possible role is that endogenous and/or extracellular myo-inositol serves as a possible source of glucose for the spermatozoa since Posternak, et al.(10) demonstrated that there is partial conversion of radioactively labelled inositol into labelled glucose. Also, extracellular myo-inositol has been found to be oxidized in small but significant amounts by both testicular (73) and ejaculated spermatozoa (75). myo-Inositol has also been proposed to be essential to the maintenance of the cellular integrity of testicular spermatozoa (11) since it was observed that myo-inositol biosynthesis was significantly

depressed three to four days after cryptorchidism of the testes; lowered myo-inositol levels in the testes were paralleled by germ cell loss.

Although the exact biochemical role of myo-inositol has yet to be clearly defined in the male reproductive system, recent evidence has suggested that myo-inositol levels in human seminal fluid may be of diagnostic importance. Lewin and Beer (76) have shown that if the myo-inositol content of seminal fluid drops from its normal value of about 500 ug/ml to a level of 100 ug/ml or less, this may be indicative of prostatitis. In contrast, if the seminal fluid contains 1000 ug/ml myo-inositol or more, it may indicate that the ejaculate consists mainly of prostatic fluid, a condition which may be associated with low fertility.

Additional Proposed Roles and Metabolism of myo-Inositol. In addition to the above roles of myo-inositol in nutrition and the male reproductive tract, a number of other functions have been proposed for this cyclitol. myo-Inositol has been shown to be a substrate for the synthesis of 6-β-galactinol in rat mammary gland and milk (77), certain indole acetic acid esters in plants (78), phytic acid and specific uronic acids and pentoses in plants (79) , and the antibiotics streptomycin (80) and bluenomycin (81). In addition, myo-inositol has been identified as a cofactor in the biosynthesis of the polysaccharides verbascose (82) and stachyose (83), functioning as an acceptor of galactosyl residues. Several investigators have demonstrated that

that myo-inositol functions as a lipotrophic agent in certain types of fatty livers (84-88). Other proposed physiological roles of myo-inositol include an inhibitor of mitotic poisons (89), a stabilizer of microtubules (90), and an effector of cell morphogenesis and cytogenesis (91). In addition, the myo-inositol containing phospholipid, phosphatidylinositol, has been suggested to be involved in post-synaptic events in the sympathetic nerves of the brain (92,93) and in the contractile mechanism of mitochondria (94).

Several investigators have studied the catabolism of myo-inositol. Charalampous originally suggested that two myo-inositol oxygenases, one producing D- and the other producing L-glucuronate existed in rat kidney homogenates (95,96). Thonet and Hoffmann-Ostenhof later refuted the claim that two oxygenases were present in the kidney and determined that only the enzyme producing D-gluronate from myo-inositol was present (97). It is now generally accepted that myo-inositol is catabolized by D-glucuronate formation through myo-inositol oxygenase, followed by formation of D-xylulose-5-phosphate production through the glucuronate-xylulose pathway (98,99). Xylulose-5-phosphate can be transformed to glycolytic intermediates through pentose phosphate interconversions and subsequently to glucose, CO_2 or other products (98).

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CHAPTER I

CHARACTERIZATION OF myo-INOSITOL CONTENT OF FETAL TISSUES FROM ETHANOL-FED AND myo-INOSITOL DEPRIVED, ETHANOL-FED DAMS

ABSTRACT

The effects of ethanol consumption by pregnant rats on fetal myo-inositol levels both during adequate and myo-inositol deprived dietary states were investigated. Ethanol feeding was used in an attempt to decrease the fetal liver cytosol $NAD^+/NADH$ ratio to determine the effect of redox state on in vivo regulation of fetal myo-inositol biosynthesis. However, ethanol consumption by dams did not significantly alter the redox state of the fetal liver under the conditions and feeding intervals tested, nor were myo-inositol levels in the fetal liver significantly changed.

Dams fed a myo-inositol adequate diet containing 10% (v/v) ethanol for a seven day interval during their gestation periods, did bear fetuses having blood plasma glucose and myo-inositol concentrations significantly lower ($P<.05$) than control levels. However, no alterations in fetal tissue myo-inositol levels were observed. In another experiment, fetal brain myo-inositol levels were significantly lower ($P<.05$) than control values in fetuses from rats maintained on 28% (v/v) ethanol and a myo-inositol free diet for a period of three to four weeks before pregnancy and throughout gestation, but no perturbations of fetal myo-inositol contents were observed in either blood plasma or other tissues analyzed.

Pregnant rats fed a myo-inositol free diet and either 30% (v/v) or 35% (v/v) ethanol during the last fourteen days of gestation had a significantly elevated ($P<.05$) plasma myo-inositol concentration (two-fold and four-fold, respectively) compared to control dams. However, in this experiment no alterations in fetal tissue or plasma myo-inositol contents were detected. The increased maternal plasma inositol levels were also paralleled by increased maternal liver free myo-inositol content, significantly higher ($P<.05$) than control values.

INTRODUCTION

The role of myo-inositol in the mammalian fetus and neonate has been under investigation by several laboratories since the original observation by Offergeld (1) that the myo-inositol concentration of human fetal blood exceeded that of maternal blood by nearly ten-fold, suggesting an important function for myo-inositol in development. Fetal blood plasma of rats (2) and other mammals (3,4) have also been shown to be from seven to eight fold higher in inositol than maternal plasma, supporting this original finding. In addition, plasma myo-inositol levels have been found to remain up to four fold higher than maternal plasma concentrations for up to sixteen days postpartum (2). Fetal myo-inositol biosynthesis was suggested as responsible for elevated fetal blood myo-inositol levels after it was observed that the cyclitol was present in the perfusate from fetal sheep liver (5) and rat fetuses or their isolated livers (6).

Later studies revealed that fetal rat liver contained high activities of 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) (2).

Although little is known about the in vivo regulation of the myo-inositol biosynthetic enzymes in fetal tissues, regulation may in part depend on the redox state of the cell cytosol. The redox state may play a role since the synthase from a number of sources has been shown to have an absolute requirement for NAD^+ in vitro, including rat mammary gland (K_m=0.5 mM) (7), rat testis (K_m=0.5 mM) (8), N. crassa (K_m=1.90 mM) (9,10), and yeast (K_m=1.50 mM) (11,12).

Since the role of the cell redox state in myo- inositol biosynthesis has not been established in vivo, especially in terms of the developing fetus and its apparently high requirement for myo-inositol, studies were conducted to alter the cytosol NAD^+/NADH ratio of the fetus. Maternal dietary ethanol was utilized in an attempt to lower the fetal cytosol NAD^+/NADH ratio and thus, the rate of fetal myo-inositol biosynthesis.

These experiments may also be of interest in the study of the biochemistry and metabolism in the clinically observed "fetal alcohol syndrome" (13). This syndrome has been recently detected in infants born to alcoholic mothers and is manifested by fetal growth retardation, morphological and cardiac anomalies, and brain dysfunction (13).

MATERIALS AND METHODS

Reagents. The following materials were obtained from the indicated sources. Choline hydrochloride, phthalylsulfathiazole, oxidized and reduced nicotinamide adenine dinucleotide (NAD^+ and NADH), and beef heart lactate dehydrogenase (EC 1.1.1.27) from Sigma Chemical Company; vitamin-free casein, myo-inositol, ∝-cellulose, Wesson mineral salt mix and vitamin fortification mixes from Nutritional Biochemical Corporation; lactalbumin hydrolysate from Gibco Diagnostics; Mazola pure corn oil from Best Foods; U.S.P. quality 100% ethanol from IMC Chemical Group, Inc.; ∝-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.

Animal Diets and Handling. For all experiments, animals were housed individually at 22 degrees in polycarbonate cages with wood shavings unless otherwise noted. The animal environment had a light cycle of 12 hours (6:00 a.m. to 6:00 p.m.).

Experiment A. In Experiment A timed pregnant Holtzman (Madison, WI) rats (250-300 g) were fed a low fat, nutritionally adequate liquid diet containing 10% ethanol (v/v) and 0.5% (w/w) myo-inositol during days 13 through 19 of their gestation periods. This diet (Table I) was a modification of the low fat liquid diet introduced by Rietz (15). A control diet, identical with the ethanol containing diet

TABLE I

Experiment A Diet Composition

Component	Concentration (% by weight)
Dextrose	39.4
Lactalbumin Hydrolysate	26.6
Ethanol[a]	22.8
Salt Solutions[b]	5.1
Mazola Corn Oil	2.3
Tween - 80	1.6
Vitamin Mix[c]	1.2
Citric Acid	0.5
myo-Inositol	0.5

[a]Control diets did not contain ethanol; a total of 138 g of dextrose was substituted for 100 ml of ethanol per liter of diet. Both ethanol containing and control diets had a caloric value of 1.58 kcal/ml.

[b]The salt solutions together consisted of (% by weight): 49.363% $CaCl_2 \cdot 2H_2O$, 39.420% KH_2PO_4, 8.890% NaCl, 1.138% $MgSO_4 \cdot 7H_2O$, 0.709% ferric ammonium citrate, 0.278% $CuSO_4 \cdot 5H_2O$, 0.137% $MnSO_4 \cdot H_2O$, 0.0580% $ZnCl_2$, 0.006% KI, and 0.0005% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

[c]The vitamin mix consisted of (g/kg): Vitamin A ester (palmitate and acetate) concentrate (200,000 U/g), 4.5; Vitamin D_3 (400,000 U/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; choline chloride, 75.0; and menaquinone, 2.25. Additional vitamins (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.

with the exception that dextrose was isocalorically substituted for ethanol, was fed to a second group of timed pregnant rats. All rats were in synchronous pregnancy, having been determined to be sperm positive on the same day by the Holtzman Company. Control rats were initially paired with alcohol fed rats on the basis of body weight and each pair was fed isocalorically throughout the experiment. Total calories consumed per day by each alcohol fed rat were fed to the corresponding control dam on the following day. On day 20 of gestation, (1 day prepartum), fetuses were excised by Caesarean section from ether anesthetized animals and examined for gross anatomical anomalies. Fetal livers from each litter were collected, pooled, and stored at -80 degrees. Fetal brains were similarly pooled and frozen. Maternal and fetal blood samples were collected in heparinized hematocrit tubes, centrifuged, and the plasma stored at -80 degrees.

Experiment B. In the second experiment 15 Holtzman virgin rats (200-220 g) were given 30 g/100 ml drinking water (27.5% ethanol v/v) and an otherwise nutritionally adequate solid diet containing no myo-inositol[1] and 0.5% (w/w) phthalylsulfathiazole for a period of 3 to 4 weeks before pregnancy in a modification of the protocol described by Tze and Lee (16). The solid diet[2] contained phthalylsulfa-

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[1] Contained less than 0.001 mg free myo-inositol per 100 g diet by gas chromatographic analysis.

[2] The diet consisted of (% by weight): 62.4% dextrose, 20.0% casein, 10.0% corn oil, 4.0% Wesson mineral salts, 2.0% myo-inositol free vitamin preparation, 1.0% -cellulose, 0.5% phthalylsulfathiazole and 0.1% choline hydrochloride. The caloric value was 4.2 kcal/g.

thiazole in order to prevent the possible contribution of of myo-inositol to the diet by intestinal flora. An isocalorically fed control group of 8 rats received the same solid diet and water. The average number of total calories consumed by the alcohol fed rats per day was fed to each of the rats in this control group on the following day. In addition a second control group of 7 rats was fed the same solid diet and water ad libitum. After 3 weeks on their respective diets, rats were mated. At mating three females were placed into a standard polycarbonate breeding cage overnight with one male of the same strain and age. Only water was available to the rats during mating. Vaginal smears were taken the following morning and no later than 12 hours after introduction to the breeding cages. The presence of spermatozoa in vaginal smears was taken as the positive criterion for pregnancy. All rats were returned to their respective cages and diets during the day and sperm negative rats were again placed into breeding cages at night for up to six successive nights or until pregnant. On day 20 of gestation, rats were ether anesthetized, fetuses quickly examined, and the fetal livers from each litter rapidly excised, frozen by immersion in liquid nitrogen, pooled, and stored at -80 degrees. Fetal brain and blood samples were collected and stored as before. Maternal blood samples were taken by heart puncture using a heparinized syringe and blood was centrifuged and plasma frozen at -80 degrees.

Experiment C. Holtzman timed pregnant rats (250-300 g) were maintained for 14 days of their gestation periods (days 6 through 19) on a solution of either 30% (v/v) or 35% (v/v) ethanol and the same myo-inositol free, phthalylsulfathiazole containing diet utilized in Experiment B. A group of control dams received the solid diet and were isocalorically pair fed with ethanol fed rats. On day 20 of gestation fetal brains, blood, and liquid nitrogen frozen fetal livers were collected and stored as before, after rapid weighing and morphological observation of each fetus. Maternal blood was collected by heart puncture and plasma obtained was stored as previously described. In addition maternal livers were removed and stored at -80 degrees.

Tissue and Plasma Free myo-Inositol and Glucose Determinations. Up to 0.1 g tissue or 40 ul plasma were deproteinized by the method of Somogyi (17) after prior addition of 30 ug or 14 ug of the internal standard ∝-methylmannoside, respectively. In all deproteinizations the ratio of tissue or plasma to the balanced 5.0% $ZnSO_4$ and 0.3 N $Ba(OH)_2$ Somogyi reagents was 1 to 7. Following centrifugation at 3200 rpm for 5 min to remove precipitated protein, supernatants were deionized by filtration through approximately 1 g of MB-3 resin, and then taken to dryness using a rotary evaporator. Tissue or plasma samples were trimethylsilylated with 150 or 100 ul respectively, using a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (5: 2: 0.5, v/v/v). After overnight incubation at room temperature, the sugar and

cyclitol derivatives were quantitated by gas-liquid chromatography according to the method of Wells, et al.(18).

Perchloric Acid Tissue Extract Preparation. Tissue extracts were prepared according to the method of Williamson, et al. (19) with several modifications. Liquid nitrogen frozen tissue was pulverized using a mortar and pestle at dry ice temperature; 1.0 g of frozen tissue was weighed into a 15 x 100 mm plastic centrifuge tube standing on dry ice and containing 1.75 ml of frozen 30% (w/v) $HClO_4$. The tubes were then placed on ice and allowed to slowly thaw until all tissue had been extracted (20 min). Following homogenization at 4 degrees using a motor driven teflon pestle, 0.69 ml of water were added and the homogenate was centrifuged at 15,000 rpm in a Sorvall SS34 rotor for 15 min to remove precipitated protein. The resulting supernatant was adjusted to pH 5-6 with 20% (w/v) KOH and the resulting $KClO_4$ precipitate removed by centrifugation as above. The extract was shaken with acid washed Florisil (activated magnesium silicate, 0.1 g/ml extract) for 30 seconds to remove endogenous fluorescence due to flavins, recentrifuged, and frozen immediately at -80 degrees.

L-Lactate Determinations. Lactate content of perchloric acid tissue extracts was determined fluorometrically through a modification of the method of Gutmann and Wahlefeld (20). Assays were conducted in 3 ml, 10 x 75 mm borosilicate Dispo culture tubes. Each reaction mixture contained the following components at the indicated final concentrations in a total

volume of 1.5 ml: 0.1 M glycine buffer, pH 10.0; 50 mM hydrazine, pH 10.0; 133 uM NAD^+, and up to 6.7 uM lactate. Initial fluorescence measurements were taken at room temperature with a Farrand Ratio Fluorometer 2 equipped with a primary filter having an excitation optimum at 340 nm and a secondary filter with an emission maximum at 460 nm. Reactions were initiated by the addition of beef heart lactate dehydrogenase (60 ug protein). Samples were incubated at room temperature for 15 minutes and the increase in fluorescence due to NADH production determined. Linearity of the assay was verified by using two different volumes of each extract. Fluorescence changes were calibrated by measuring the increase in fluorescence with the addition of known amounts of lactate and NADH.

Pyruvate Determinations. Pyruvate levels of perchloric acid tissue extracts were determined fluorometrically by the method of Passonneau and Lowry (21) with minor modifications. Each reaction tube contained the following components at the indicated final concentrations in a total volume of 1.5 ml: 150 mM imidazole buffer, pH 7.0; 3.33 uM NADH, and up to 2.0 uM pyruvate. After determining the initial fluorescence using the same conditions as described in the lactate assay, reactions were initiated by the addition of beef heart lactate dehydrogenase (5.75 ug protein). After incubating at room temperature for 20 minutes, the decrease in NADH fluorescence was measured. Fluorescence changes were calibrated by measuring both the decrease in fluorescence with the addition of a known amount of pyruvate and

the increase in fluorescence with the addition of a known quantity of NADH.

Calculation of NAD^+/NADH Ratio. The cytosolic NAD^+/NADH ratio was calculated by the method of Krebs, et al.(22). Upon rearranging the equilibrium constant expression for the cytosolic lactate dehydrogenase system, the following relationship is obtained:

$$NAD^+/NADH = \text{pyruvate/lactate} \times 1/K,$$

where K is equal to 1.11×10^{-4} at pH 7.0, ionic strength 0.25, and 38 degrees, and takes into account the hydrogen ion concentration.

Statistics. Statistical analyses were carried out using either two-tailed t-tests or paired t-tests as indicated (23).

RESULTS

Experiment A. Table II presents data on the litter size, maternal diet consumption, and maternal weight profile for the 10% (v/v) ethanol fed and pair fed control dams of Experiment A. Caloric intake for both groups was comparable. During the 7 day experiment (diets were fed during days 13 through 19 of gestation), ethanol fed rats consumed 25.7 $\pm$ 3.1 ethanol calories/day, which accounted for 34.9 $\pm$ 0.4% of the total calories consumed. No significant differences were noted for either the average maternal weight increase during the experiment or for the average litter size of the two groups. In addition, 10% (v/v) ethanol feeding for the 7 day interval did not appear to affect the number of successful pregnancies; 9 out of 12 sperm positive ethanol fed rats and 8 out of 12 control diet fed sperm positive rats actually were pregnant. There was no evidence of resorptions from both groups not bearing fetuses; most likely these rats were not pregnant initially.

Table III shows the myo-inositol levels of selected fetal tissues and blood plasma, as well as fetal plasma glucose concentrations for the 10% (v/v) ethanol fed and control diet fed groups. Plasma myo-inositol concentrations were 1.6 fold lower ($P<0.1$) in fetuses from the ethanol fed dams than for fetuses from control dams. However, no significant differences existed between the two fetal groups with respect to free myo-inositol levels in brain

TABLE II

Experiment A: Litter Size, Diet Consumption, and Weight Profile of Pregnant Ethanol Fed and Pair Fed Control Rats[a]

Experimental Group	Total Calories Consumed/Day	Ethanol Calories Consumed/Day	Initial Body Weight (g)	Final Body Weight (g)	Weight Increase (g)	Litter Size
10% (v/v) Ethanol Fed	73.6 ± 9.0	25.7 ± 3.1	304.3 ± 15.6	340.3 ± 21.6	36.0 ± 14.4	10.3 ± 3.0[b]
Pair Fed Controls	68.1 ± 12.5	---	302.6 ± 18.7	328.4 ± 19.2	25.9 ± 15.5	11.3 ± 2.1[c]

[a]All values are for only those rats which bore fetuses and are expressed as the mean ± standard deviation. Ethanol fed rats received a liquid diet containing 10% (v/v) ethanol during days 13 through 19 of their gestation periods. Control rats were isocalorically fed a comparable liquid diet during this same interval in which dextrose was substituted for ethanol. Both liquid diets contained 0.5% myo-inositol. All other conditions were as described in the Materials and Methods section.

[b]n = 9

[c]n = 8

TABLE III

Experiment A: Selected Fetal Tissue myo-Inositol and Plasma Glucose Concentrations[a]

Tissue	Fetuses of 10% Ethanol Fed Dams	Fetuses of Control Diet Fed Dams
Brain	3.38 ± 1.04	3.42 ± 1.49
Liver	0.410 ± 0.140	0.360 ± 0.080
Plasma	0.194 ± 0.084[b]	0.304 ± 0.130
Plasma (glucose)	1.28 ± 0.16[b]	2.47 ± 0.96

[a]Brain and liver myo-inositol values are expressed as umoles/g wet weight. Plasma myo-inositol and glucose levels are expressed as mmoles/l. All values are the mean ± standard deviation. Inositol and glucose assays were as outlined in the Methods section. n = 8, except for plasma glucose where n = 7.

[b]Significantly different from controls ($P<.01$) by two-tailed paired t-test.

and liver. Fetal plasma glucose concentrations were 1.9 fold lower ($P<.01$) in the ethanol fed group than for the control diet fed group, paralleling the difference in plasma myo-inositol concentrations. Although not shown in Table III maternal plasma myo-inositol and plasma glucose concentrations were not significantly different for the ethanol fed and control diet fed dams. Ethanol fed and control diet fed maternal plasma myo-inositol levels were 68.2 ± 18.1 μM (n=9) and 74.3 ± 20.4 μM (n=8), respectively. Plasma glucose from ethanol fed dams was 8.50 ± 1.60 mM (n=9) and from control diet fed rats 7.36 ± 2.3 mM (n=8).

Experiment B. Table IV presents data concerning the pregnancy rate, litter size, and diet consumption of ethanol fed and control rats of Experiment B. In this experiment the effects of a long-term, chronic ethanol consumption on fetal myo-inositol metabolism were determined. As shown, rats maintained on 27.5% (v/v) ethanol and a myo-inositol free diet (as determined by gas-liquid chromatography) had a lower percentage of successful pregnancies as compared to group fed and ad libitum control rats under the same mating conditions. In addition ethanol fed dams had a significantly smaller ($P<.05$) litter size than the group fed control dams. Although daily caloric intakes by the alcoholic and the isocalorically fed control groups were comparable, the average weight gain during the experiment for the ethanol fed rats was only 55% of that recorded for the group fed controls, probably a reflection of dehydration

TABLE IV

Experiment B: Pregnancy Rate, Litter Size, and Diet Consumption for Ethanol Fed and Control Rats[a]

	Ethanol Fed	Group Fed Controls	Ad Libitum Fed Controls
Successful Pregnancies	11 (73.3%)	7 (87.5%)	6 (85.7%)
Litter Size	9.4 ± 1.9[b]	12.4 ± 2.2	11.2 ± 1.5
Weight Gain (g)	84.5 ± 13.2	153.0 ± 18.2	150.7 ± 29.6
Total Calories Consumed/Day	93.0 ± 14.1	100.4 ± 9.1	N.D.
EtOH Calories Consumed/Day	23.2 ± 2.7	---	---

[a]Ethanol fed dams received a solution of 27.5% (v/v) ethanol as described in the Methods section. All values are expressed as the mean ± standard deviation.

[b]Significantly different from group fed controls ($P<.05$) using two-tailed t-test.

and the smaller average litter size of the ethanol fed dams. The ethanol fed rats consumed an average of 23.2 ± 2.7 ethanol calories/day or 24.9 ± 2.9% of their average total daily calories.

The maternal body weight profile for the three groups of experimental animals used in Experiment B is shown in Figure 1. Ethanol fed rats lost weight during the first five days of their diet; however, after this initial loss, they gained weight at approximately the same rate as did the isocalorically fed control rats, both before and during pregnancy. Ad libitum fed control rats showed a rate of weight gain slightly larger than the control group isocalorically fed with the ethanol fed rats.

The free myo-inositol content of several fetal tissues and plasma from ethanol fed and control dams is presented in Table V. Fetal brain myo-inositol levels were significantly lower ($P<.05$) in fetuses from ethanol fed dams than in fetuses from isocalorically fed control dams. No significant differences were found between the experimental groups with respect to fetal liver or fetal plasma myo-inositol levels.

Also shown in Table V are the cytosolic ratios of $NAD^+/NADH$ for fetal livers from the three experimental groups, as determined by the fetal hepatic substrate levels of the NAD^+-linked lactate dehydrogenase system. Fetal liver from ethanol fed dams showed a slight reduction in the $NAD^+/NADH$ ratio as compared to the isocaloric control group; however, differences were not significant at the

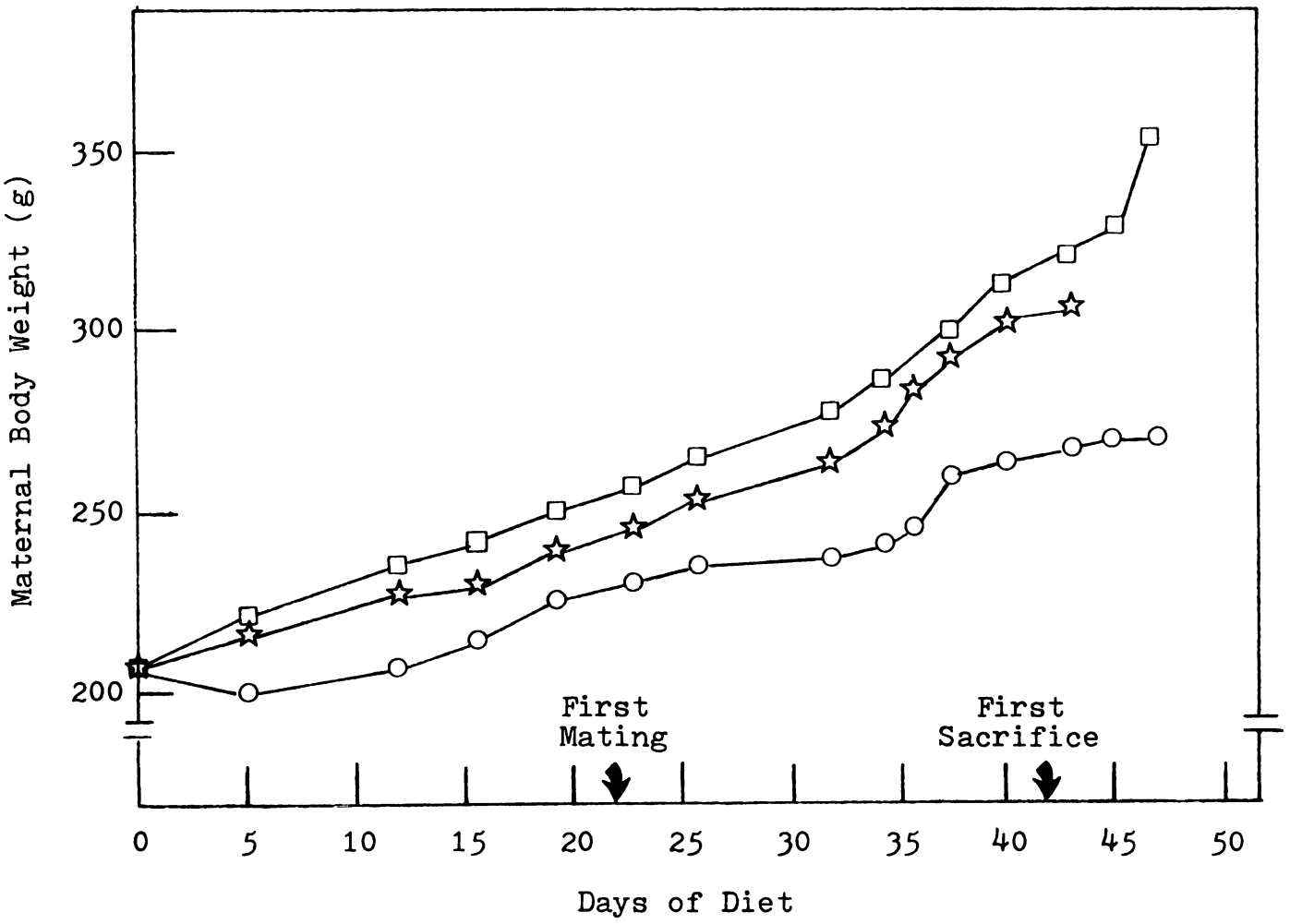

Figure 1: Maternal Weight Profile (Experiment B).
○--27.5% (v/v) ethanol fed dams; ☆--group fed control dams (isocalorically fed to ethanol fed dams); □--ad libitum fed control dams. Each point represents the average weight for 11 EtOH fed, 7 group fed, and 6 ad libitum fed animals. All diets and conditions were as described in the Methods section.

TABLE V

Experiment B: Selected Fetal Tissue myo-Inositol Levels and Fetal Liver Cytosolic $NAD^+/NADH$ Ratios[a]

Tissue	Ethanol Fed	Group Fed Controls	Ad Libitum Fed Controls
Brain myo-inositol (umoles/g)	4.07 ± 0.40[b] (n = 11)	5.01 ± 0.34 (n = 6)	5.14 ± 0.94 (n = 5)
Liver myo-inositol (umoles/g)	0.491 ± 0.082 (n = 6)	0.454 ± 0.178 (n = 6)	0.508 ± 0.258 (n = 5)
Plasma myo-inositol (mmoles/l)	0.434 ± 0.080 (n = 6)	0.569 ± 0.139 (n = 7)	0.475 ± 0.104 (n = 5)
Liver lactate (umoles/g)	4.79 ± 0.96 (n = 5)	4.65 ± 0.74 (n = 3)	5.18 ± 1.04 (n = 5)
Liver pyruvate (umoles/g)	0.077 ± 0.017 (n = 5)	0.126 ± 0.038 (n = 3)	0.152 ± 0.029 (n = 5)
Liver Cytosol[c] $NAD^+/NADH$	146.3 ± 18.9	227.0 ± 74.3	266.3 ± 105.2

[a]Ethanol fed dams received a solution of 27.5% (v/v) ethanol. All dams were fed a myo-inositol free diet as described in the Methods section.

[b]Statistically different from control groups ($P < .05$) using two-tailed t-test.

[c]The K for the lactate dehydrogenase reaction was taken as 1.11×10^{-4}.

P=.05 level.

Although fetuses were not weighed prior to dissection, it was generally observed that fetuses from the ethanol fed dams were smaller than control fetuses from either group. In addition, fetuses from alcohol fed mothers appeared more emaciated than control fetuses. No gross malformations or resorptions were observed in fetuses from any of the experimental animals although some ethanol fed dams exhibited fatty livers.

Experiment C. The effects of ethanol consumption (either 30% (v/v) or 35% (v/v)) on fetal tissue myo-inositol metabolism were investigated during the latter part of pregnancy (days 6 through 19). Table VI presents data on ethanol consumption and litter size of pregnant rats in this experiment, along with the corresponding average body weights and hepatic cytosolic NAD^+/NADH ratios of fetuses from these dams. As shown, ethanol consumption was comparable for both the 30% and the 35% ethanol fed dams. Ethanol calories amounted to 33.5% and 30.7% of the total calories consumed per day by the 30% and 35% ethanol fed dams, respectively. No significant differences were observed in the average litter sizes of ethanol fed dams in comparison to those of isocalorically pair fed or ad libitum fed control dams. However, alcohol fed dams bore fetuses having significantly lower ($P<.01$) body weights than fetuses for either pair fed or ad libitum fed control dams. In addition, fetuses from alcohol fed dams appeared to be of smaller proportions and generally emaciated compared to control fetuses; however,

TABLE VI

Experiment C: Ethanol Consumption by Pregnant Rats, Litter Size, Fetal Body Weights, and Fetal Liver Cytosolic $NAD^+/NADH$ Ratios[a]

Parameter	30% (v/v) Ethanol Fed	Pair Fed Controls	35% (v/v) Ethanol Fed	Pair Fed Controls	Ad Libitum Fed Controls
Litter Size	11.8 ± 1.0 (n = 6)	10.7 ± 2.6 (n = 6)	11.3 ± 1.6 (n = 6)	12.0 ± 2.2 (n = 6)	9.2 ± 1.6 (n = 5)
EtOH Calories Consumed/Day	35.5 ± 9.5 (n = 6)	---	31.0 ± 4.6 (n = 6)	---	---
Fetal Body Weight (g)	3.51 ± 0.61[b] (n = 71)	4.04 ± 0.31 (n = 64)	2.62 ± 1.04 (n = 69)	4.00 ± 0.45 (n = 64)	4.54 ± 0.42 (n = 46)
Fetal Liver Lactate (umoles/g)	7.90 ± 2.44 (n = 3)	6.42 ± 1.08 (n = 3)	7.10 ± 1.34 (n = 4)	5.97 ± 0.215 (n = 4)	5.10 ± 0.22 (n = 2)
Fetal Liver Pyruvate (umoles/g)	0.066 ± 0.021 (n = 3)	0.094 ± 0.019 (n = 3)	0.074 ± 0.012 (n = 4)	0.083 ± 0.023 (n = 4)	0.120 ± 0.001 (n = 2)
Fetal Liver Cytosolic NAD/NADH	82.0 ± 37.3 (n = 3)	132.2 ± 8.5 (n = 3)	97.5 ± 29.7 (n = 4)	125.4 ± 38.9 (n = 4)	211.5 ± 10.3 (n = 2)

[a]All animals were fed a myo-inositol free diet as described in the Methods section. The K for lactate dehydrogenase was taken to be 1.11×10^{-4}.

[b]Statistically different from control groups ($P < .01$) using two-tailed paired t-test.

no gross morphological anomalies or resorptions were noted. It was also observed that alcohol fed dams had both fatty livers and fatty placentas in most cases. As shown in Table VI, no significant differences were found between the liver cytosolic redox states of fetuses from ethanol fed dams and those fetuses from control dams.

The myo-inositol content of maternal and fetal tissues are presented in Table VII. Maternal plasma from 30% (v/v) and 35% (v/v) ethanol fed dams was two fold and four fold higher in myo-inositol content, respectively, than plasma from either pair fed or ad libitum fed control rats. Maternal liver myo-inositol was significantly higher ($P<.05$) in 30% (v/v) ethanol fed dams compared to isocalorically fed control dams. Liver myo-inositol was also significantly higher ($P<.02$) for 35% (v/v) ethanol fed dams than either pair fed or ad libitum fed control dams. No significant differences were observed, however, in fetal plasma, liver, or brain myo-inositol contents between alcohol fed and control groups.

TABLE VII

Experiment C: Fetal and Maternal Tissue and Plasma *myo*-Inositol Levels[a]

Tissue	30% (v/v) Ethanol Fed	Pair Fed Controls	35% (v/v) Ethanol Fed	Pair Fed Controls	*Ad Libitum* Fed Controls
Maternal Plasma (umoles/l)	53.5 ± 14.4[b] (n = 6)	25.8 ± 8.6 (n = 6)	97.1 ± 32.1[b] (n = 5)	22.3 ±5.1 (n = 5)	23.9 ± 8.3 (n = 5)
Maternal Liver (umoles/g)	0.354 ± 0.014[c] (n = 4)	0.307 ± 0.031 (n = 4)	0.408 ± 0.042[d] (n = 5)	0.325 ± 0.045 (n = 5)	0.314 ± 0.043 (n = 5)
Fetal Plasma (mmoles/l)	0.414 ± 0.144 (n = 6)	0.347 ± 0.181 (n = 6)	0.403 ± 0.042 (n = 4)	0.438 ± 0.085 (n = 4)	0.300 ± 0.137 (n = 4)
Fetal Liver (umoles/g)	0.446 ± 0.134 (n = 5)	0.424 ± 0.099 (n = 5)	0.535 ± 0.109 (n = 3)	0.424 ± 0.078 (n = 3)	0.382 ± 0.038 (n = 2)
Fetal Brain (umoles/g)	3.914 ± 1.04 (n = 6)	3.251 ± 0.782 (n = 6)	4.480 ± 1.15 (n = 6)	3.803 ± 1.15 (n = 6)	4.568 ± 0.793 (n = 5)

[a]All values are expressed as the mean ± standard deviation. The paired t-test was used for statistical analyses. All diets and experimental conditions were as outlined in the Methods section.

[b]Significantly different from controls at $P<.01$.

[c]Significantly different from pair fed control at $P<.05$.

[d]Significantyl different from controls at $P<.02$

DISCUSSION

Feeding of ethanol to pregnant rats at the dosages and durations used in this study had a minimal effect on the fetal liver cytosolic NAD^+/NADH ratio and fetal liver myo-inositol content. However, ethanol consumption was shown to perturb fetal brain and plasma myo-inositol levels under certain conditions.

The absence of an ethanol effect on the fetal cytosolic redox state does not imply a placental barrier to ethanol since this drug has been demonstrated to be present in fetal blood at the same levels as in maternal blood (24-28). However, it is possible that the majority of ethanol consumed was metabolized by the maternal liver, thereby significantly decreasing ethanol exposure to the fetus. Furthermore, alcohol dehydrogenase activity of fetal rat liver is reported to be low; approximately 150 nmoles/min/g at 3 days before term, or one-sixth the activity found in adult rat liver (29). Therefore, depletion of the cytosolic NAD^+ pool through the conversion of NAD^+ to its reduced form during the alcohol dehydrogenase catalyzed oxidation of ethanol to acetaldehyde probably does not occur to a significant extent in fetal liver or other fetal tissues in vivo. Although the NAD^+ linked acetaldehyde dehydrogenase activity of fetal rat liver homogenates is reported to be fairly high (49% of the activity found in adult rat liver), several studies have shown that the majority of maternal plasma acetaldehyde is oxidized

by the placenta (30-32). Since the oxidation of acetaldehyde in fetal liver is limited, a significant decrease in the cytosolic NAD^+/NADH ratio due to the action of acetaldehyde dehydrogenase would not be expected. Also, since acetaldehyde dehydrogenase is mainly a mitochondrial enzyme, acetaldehyde oxidation would be expected to have only a minimal effect on the cytosolic redox state.

It has been demonstrated *in vitro* by Wells, *et al.* that the activity of D-glucose-6-phosphate: L-*myo*-inositol-1-phosphate synthase from adult rat brain is decreased by 10% when the NAD^+/NADH ratio is reduced two fold from its optimal ratio (33). Further, it was shown that a ten fold reduction in the NAD^+/NADH ratio from the optimal ratio resulted in only a 30% inhibition of *myo*-inositol formation. While no direct comparison can be drawn between the adult rat brain synthase and the fetal liver synthase, it is probable that a considerable reduction in the NAD^+/NADH ratio is required for a significant decrease in *in vivo* *myo*-inositol biosynthesis.

Plasma *myo*-inositol concentrations were observed to be significantly lower than control levels in fetuses from rats fed 10% (v/v) ethanol and a nutritionally adequate diet containing 0.5% (w/w) *myo*-inositol during 7 days of their pregnancy (Experiment A). However, this reduction in fetal plasma inositol was not accompanied by a decrease in the *myo*-inositol content of any of the fetal tissues measured. Plasma *myo*-inositol levels were not different from control concentrations in fetuses from rats fed 27.5%

(v/v) ethanol for 3 to 4 weeks prior to and throughout pregnancy and a solid diet free of myo-inositol (Experiment B). However, fetuses from ethanol fed dams had lower myo-inositol levels in the brain compared to control animals. In a similar experiment in which either 30% (v/v) or 35% (v/v) ethanol was fed to pregnant rats for 14 days during their gestation periods along with a myo-inositol free diet, no significant differences were observed in the fetal plasma or tissue myo-inositol levels (Experiment C). However, maternal plasma and liver free myo-inositol concentrations were significantly greater than control values. These observations may possibly be explained in terms of ethanol effects on placental transport of myo-inositol, transport of myo-inositol from fetal tissues to fetal plasma, phosphatidylinositol metabolism, or myo-inositol catabolism.

Further work in this research area might emcompass defining the effects of ethanol on each of these processes above. In addition, the mechanism of myo-inositol transport across the placenta remains to be elucidated. Placental transfer of myo-inositol from maternal to fetal blood is probably of major importance since it has been estimated to supply approximately 40% of the myo-inositol present in the fetal plasma, the remaining 60% originating from fetal tissue biosynthesis of the cyclitol (34). Since placental transport of myo-inositol must occur across a concentration gradient, such a process would most likely involve active transport.

Investigation of the redox state regulation of myo-

inositol biosynthesis in fetal tissue will have to be approached through means other than ethanol feeding due to several complications. These include low fetal ethanol exposure due to metabolism of ethanol by the maternal liver, low ethanol oxidizing capacity of the fetal liver, cytotoxic effects of ethanol, and alteration of a significant number of metabolic processes with ethanol feeding.

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CHAPTER II

myo-INOSITOL BIOSYNTHESIS IN THE RAT TESTIS DURING HYPERGLYCEMIA OR ETHANOL FEEDING

ABSTRACT

The effects of streptozotocin induced hyperglycemia and prolonged ethanol feeding on de novo myo-inositol biosynthesis in the rat testis were examined. Acute hyperglycemia was evident in rats 72 hours after streptozotocin administration. Testis myo-inositol contents were 2.7 fold higher in diabetic animals than in control rats injected with citrate buffer. No changes were observed in the specific activities of the myo-inositol biosynthesizing enzymes; however, hyperglycemic rats displayed elevated testis glucose and glucose-6-phosphate levels, approximately 4 fold and 2 fold in excess of control values, respectively. Insulin treatment of diabetic rats resulted in the lowering of plasma glucose, testis glucose, and testis glucose-6-phosphate to normal or subnormal levels, but myo-inositol levels remained significantly elevated compared to control animals, although slightly lower than that observed for untreated diabetic rats. Testis glucose and glucose-6-phosphate levels increased significantly 10 hours and 12 hours after streptozotocin injection, respectively. However, testis myo-inositol content lagged and did not increase appreciably until 24 hours following injection of the drug. Streptozotocin diabetic rats had a significantly decreased testis cytosolic NAD^+/NADH ratio compared to control

animals 72 hours after injection.

Feeding of 40% ethanol to male rats for a three day period resulted in a small but significant decrease in testis glucose, glucose-6-phosphate, and myo-inositol levels compared to control animals, but no significant alterations in the testis cytosolic redox state were observed. Subsequent work utilizing an isocaloric paired feeding model failed to show perturbations in the testis myo-inositol level or testis redox state of rats fed 40% ethanol for two weeks.

The potential role of testis hexokinase distribution in the regulation of myo-inositol biosynthesis in normal, diabetic, and ethanol intoxicated rats was investigated. No significant differences in testis hexokinase distribution or in the kinetic characteristics of the soluble and particulate hexokinase activities were observed.

Testicular sperm counts and tubulin polymerization in streptozotocin diabetic rats were not significantly different from control values.

INTRODUCTION

Eisenberg and Bolden (1) were the first to describe an active system in the mammalian testis for the synthesis of myo-inositol and postulated that the process occurred at the level of the seminiferous tubule. Later Voglmayr and White demonstrated that testicular spermatozoa were capable of synthesizing myo-inositol but indicated that it was unlikely that spermatozoa were the only site in the testis for the biosynthesis of the cyclitol (2). In spite of a high bio-

synthetic rate the testis stores little myo-inositol, while the epididymis, which possesses only a slight capacity for synthesis, maintains a much higher concentration of myo-inositol (3). Subsequent workers described a testis-blood barrier for myo-inositol which did not exist for the epididymis or accessory sex organs (4,5).

The synthase catalyzed conversion of glucose-6-phosphate to inositol-1-phosphate has been identified as the rate limiting step in myo-inositol biosynthesis (6) and therefore, a probable site for metabolic regulation of the pathway. In fact, several in vitro inhibitors of the synthase from several sources have been reported including NADH (7-9), pyrophosphate (10), 2-deoxy-D-glucose-6-phosphate (11), and 5-thio-D-glucose-6-phosphate (12). In vivo studies with 2-deoxy-D-glucose demonstrated that this glucose analog was converted to the 6-phosphate in mouse testis and liver, and myo-inositol levels were significantly decreased in both tissues (12). While myo-inositol has been detected in the urine of diabetics (13,14), little is known of the effects of hyperglycemia on in vivo myo-inositol biosynthesis in the testis, a tissue in which glucose uptake is insulin independent (15). Therefore, experiments were conducted using streptozotocin diabetic rats to determine the effects of hyperglycemia on testicular glucose, glucose-6-phosphate, and myo-inositol levels. In addition, the effects of ethanol induced hypoglycemia and a decreased cytosolic NAD^+/NADH ratio on myo-inositol metabolism in the rat testis were investigated. These studies are of interest since alteration of testicular myo-inositol bio-

synthesis may be related to impairment of male reproductive function in mammals during diabetes mellitus (16-22) and testicular atrophy and infertility in man during alcoholism (23-28).

MATERIALS AND METHODS

Reagents. The following reagents were obtained from the indicated sources. Streptozotocin was the generous gift of the Upjohn Co., Kalamazoo, MI. Iletin (bovine zinc-insulin) was obtained from the Eli Lilly Co.; colchicine (3 Ci/mmole) from New England Nuclear; dimethylsulfoxide (DMSO) from Fisher Scientific Co.; R. speroides β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), beef heart lactate dehydrogenase (EC 1.1.1.27), yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49), E. aerogenes citrate lyase (EC 4.1.3.6), pig heart malate dehydrogenase (EC 1.1.1.37), yeast hexokinase (EC 2.7.1.1), oxidized and reduced nicotinamide adenine dinucleotide (NAD^+, NADH), oxidized and reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$, NADPH), ethylene glycol bis (β-aminoethyl ether) N, N, N',N'-tetra-acetic acid (EGTA), sodium guanosine triphosphate (GTP), and N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) from Sigma Chemical Co.; USP quality 100% ethanol from IMC Chemical Group Inc.; α-methylmannoside 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. L-myo-inositol-1-phosphate was previously prepared in this laboratory.

Animals and Diets. Male rats (200-225 g) of the Holtzman strain (Madison, WI) were used in all studies. Animals were fed a standard solid diet of Wayne Lab Blox (Allied Mills, Chicago, IL) and water ad libitum unless otherwise noted. Exceptions included an experiment in which a group of rats was maintained on the standard solid diet and a solution of 40% (v/v) ethanol for 3 days. In another ethanol study control rats receiving the standard solid diet and water were pair fed with rats receiving the same solid diet and a solution of 40% (v/v) ethanol for 14 days.

Administration of Streptozotocin. Within 20 min before use, a stock solution of 65 mg/ml streptozotocin in 100 mM acetate buffer, pH 4.5, was prepared and kept on ice. Hyperglycemia was induced in male rats by injection of streptozotocin (65 mg/kg) into the saphenous vein of ether anesthetized animals using a 1 ml plastic syringe equipped with a 30 gauge needle. Control animals received injections of citrate buffer (1 ml/kg) under otherwise identical conditions. Leg incisions were closed with metal wound clips following all injections.

Administration of Insulin. Streptozotocin diabetic rats to be injected with insulin were initially proven hyperglycemic by analysis of blood glucose in samples collected in heparinized capillary tubes from tail vein incisions (see below for glucose assay). Exactly 72 hours after streptozotocin injection, diabetic rats were divided into two groups; one group receiving 40 units Iletin/kg (i.p.) and a second group receiving an i.p. injection of an equal volume of physiological

saline. Following an interval of 4 hours after insulin or saline injection, all rats were sacrificed and tissues collected as described below.

Collection of Tissue and Plasma for myo-Inositol and Metabolite Analysis. Rats were weighed and placed under light ether anesthesia. The right testis of each animal was rapidly excised, the epididymis and connective tissue detached, and the testis immersed in liquid nitrogen within 15 seconds. The left testis was removed and placed on ice. In some experiments, a portion of the liver was freeze-clamped using liquid nitrogen cooled tongs and the tissue was immersed immediately in liquid nitrogen. Blood was collected by heart puncture using a syringe previously rinsed with heparinized saline. Plasma was prepared from whole blood kept at 4 degrees by centrifugation at 3200 rpm in a Brinkman desk top centrifuge.

Plasma and Tissue Glucose Assays. Plasma glucose levels were determined on 10 ul samples using a Gilford 3500 Semi-Automated Computer-Directed Analyzer and a Worthington/Gilford reagent kit containing the enzymes hexokinase and glucose-6-phosphate dehydrogenase. Tissue glucose determinations were conducted on perchloric acid extracts of liquid nitrogen frozen tissue using the same method and including suitable blanks to compensate for endogenous light absorption at 340 nm by the extracts.

Perchloric Acid Tissue Extract Preparation and Assays for Metabolites. Perchloric acid extracts of liquid nitrogen

frozen tissues were prepared as previously described in Chapter I. Glucose-6-phosphate, ATP, and citrate were analyzed by the methods of Lowry and Passonneau (29). D-(-)-3-hydroxybutyrate and acetoacetate were determined by the method of Williamson and Mellanby (30). Pyruvate and L-lactate were analyzed as previously described in Chapter I.

Calculation of Cytosolic and Mitochondrial NAD^+/NADH Ratios. The calculation of the cytosolic and mitochondrial NAD^+/NADH ratios was done according to the method of Krebs, et al.(31) employing the lactate dehydrogenase and β-hydroxybutyrate dehydrogenase systems. In general,
NAD^+/NADH = (oxidized substrate)/(reduced substrate) x 1/K,
where $K = 1.11 \times 10^{-4}$ for the lactate dehydrogenase system, and $K = 4.93 \times 10^{-2}$ for the β-hydroxybutyrate dehydrogenase system at 38 degrees, pH 7.0, and ionic strength 0.25.

Plasma and Tissue myo-Inositol Determinations. The myo-inositol content of plasma and tissue samples was determined by gas-liquid chromatography by the method of Wells (32). α-Methylmannoside was used as the internal standard in all determinations.

Preparation of myo-Inositol Biosynthesizing Enzymes from Rat Testis. Synthase was prepared according to the procedure of Barnett, et al.(33) with minor modifications. Heat treatment of the homogenate at 60 degrees was extended from 2 minutes to 10 minutes to insure inactivation of non-specific phosphatases and phosphoglucoisomerase. The additional 8 minutes of heat treatment was found to give

approximately 2 fold greater yields of the cyclase and phosphatase. This step was conducted prior to centrifugation at 105,000 x g. The resolubilized 40% ammonium sulfate precipitate was dialyzed overnight at 4 degrees against 4 l of 50 mM Tris-acetate, pH 7.5, and 1 mM β-mercaptoethanol, and then centrifuged at 14,500 x g for 15 minutes. The supernatant was assayed immediately for synthase activity. The phosphatase was obtained from the supernatant fraction of synthase preparations by adjusting the ammonium sulfate concentration from 40% to 60% saturation, dialyzing the resolubilized precipitate against 4 liters of the buffer above, and recentrifugation of the dialysate.

D-Glucose-6-Phosphate: L-myo-Inositol-1-Phosphate Synthase Assay. The synthase assay was performed according to the method of Barnett, et al (33). Inorganic phosphate liberated from L-myo-inositol-1-phosphate by sodium periodate treatment was assayed as described below. One unit of synthase activity was defined as 1 nmole of myo-inositol-1-phosphate produced per hour at 37 degrees.

L-myo-Inositol-1-Phosphate Phosphatase Assay. The phosphatase was assayed using the method of Eisenberg (6). The reaction mixture was incubated at 37 degrees for 30 minutes and was contained in a final volume of 0.5 ml 40 mM Tris-acetate, pH 7.4; 100 mM KCl, 3 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM L-myo-inositol-1-phosphate, and 15 ug of protein. The reaction was terminated after 30 minutes by addition of 0.25 ml 20% TCA. After removal of precipitated protein by centrifugation,

a 0.5 ml aliquot was analyzed for inorganic phosphate as described below. A blank in which TCA was added prior to the addition of the enzyme preparation was employed to compensate for inorganic phosphate released by the action of TCA and other background inorganic phosphate. One unit of phosphatase activity was defined as 1 umole of phosphate liberated from L-myo-inositol-1-phosphate per hour at 37 degrees.

Determination of Inorganic Phosphate. Inorganic phosphate was assayed using a modification of the method of Ames (34). A total of 2.1 ml of phosphate color reagent containing one part 10% (w/v) ascorbic acid and six parts of 0.42% ammonium molybdate·$4H_2O$ in 1.0 N H_2SO_4 were added to up to 0.9 ml of the sample to be assayed. After incubation at 45 degrees for 20 min and cooling to room temperature, absorbance due to the reduced phosphomolybdate complex was measured at 700 nm using a Gilford Model 300 spectrophotometer.

Protein Determinations. Protein was determined by the method of Lowry, et al.(35) using bovine serum albumin as the standard.

Pair Feeding of Ethanol Fed and Control Rats. In order to insure a comparable caloric intake for ethanol fed and control rats, a pair feeding model was implemented in one experiment. Rats were fed a 40% (v/v) solution of ethanol (2.23 kcal/ml) and a commercial solid diet (Wayne Lab Blox, Chicago, IL) (2.97 kcal/g) ad libitum for two weeks and total and ethanol caloric intakes were calculated daily. Ethanol solutions were made available to animals through pint-sized

drinking bottles and suitable control bottles were included to compensate for spillage and evaporation. Control rats were initially paired with ethanol fed rats on the basis of body weight and maintained on water and the same commercial solid diet. However, caloric intake was restricted for control animals to the equivalent number of calories consumed on the previous day by the corresponding ethanol fed rat.

Hexokinase Preparation. The preparation of hexokinase from rat testes was a modification of the method of Wells, et al. (36). Testes from ether anesthetized rats were rapidly removed, submerged in cold 0.25 M sucrose containing 1mM β-mercaptoethanol, immediately minced with scissors, and completely homogenized for 30 seconds at 65 volts using a Tekmar homogenizer. The time between excision of tissue and the initiation of homogenization was less than 20 seconds. Samples were taken from either normal, streptozotocin diabetic or ethanol injected rats. For diabetic rats, streptozotocin was administered 72 hours before sacrifice. Ethanol injected rats received 10% (v/v) ethanol at a dosage of 2 g EtOH/kg intraperitoneally and were sacrificed 25 minutes after injection. An aliquot of the crude homogenate was assayed for hexokinase activity and the remainder was centrifuged at 40,000 x g for 10 minutes which provided two fractions: the supernant containing soluble hexokinase and the particulate, which was calculated from the difference between soluble and crude homogenate activities.

Hexokinase Assay. Determination of hexokinase activity was by a modification of the procedure of Hernandez and Crane (37). Each reaction mixture contained 3.3 mM glucose, 6.7 mM ATP, 6.7 mM $MgCl_2$, 40 mM potassium HEPES, pH 7.5; 10 mM 1-thioglycerol, 0.64 mM $NADP^+$, and 1 unit of glucose-6-phosphate dehydrogenase in a total volume of 1.0 ml. The reaction was initiated by adding an aliquot of sample. NADPH formation was followed at 340 nm using a Gilford 2400-S recording spectrophotometer at 30 degrees. One unit of hexokinase was defined as 1 umole of NADPH formed per minute.

Kinetic Studies. Kinetic studies were conducted on soluble and particulate hexokinase preparations from control rat testis. Rates were determined by monitoring the formation of NADPH produced through the coupled hexokinase and glucose-6-phosphate dehydrogenase reactions with time. The K_ms for ATP and glucose of the hexokinase preparations were calculated from the appropriate kinetic data using computer generated Hill plots.

Sperm Counts. Sperm counts were conducted according to the method of Kirton, et al.(38).

Determination of Total and Polymerized Tubulin in Testes. Polymerized and depolymerized forms of tubulin in testes were quantitated through the colchicine-binding assay of Pipeleers, et al.(39). The depolymerized tubulin fraction was prepared by homogenizing 0.3 g testes in 5 ml of a microtubule stabilizing solution containing 50% glycerol, 5% DMSO, 0.5 mM GTP, 0.5 mM $MgCl_2$, and 0.5 mM EGTA in 10 mM phosphate buffer, pH 6.95, followed by centrifugation at

100,000 x g for 45 min at 30 degrees. The amount of free depolymerized tubulin was then determined by assaying an aliquot of the resulting supernatant. The polymerized tubulin fraction was obtained by resuspending the high speed pellet in a tubulin depolymerizing solution containing 0.25 M sucrose, 0.50 mM $MgCl_2$, and 0.50 mM GTP in 10 mM phosphate buffer, pH 6.95, followed by centrifugation at 100,000 x g for 45 minutes at 4 degrees. The resulting supernatant was assayed by the colchicine-binding method to determine polymerized tubulin content.

Statistics. Unless otherwise noted, statistical analyses were performed using the two-tailed t-test (40).

RESULTS

Effects of Streptozotocin Induced Diabetes and Subsequent Intraperitoneal Insulin Injection on Testes myo-Inositol Levels. The effects of streptozotocin induced hyperglycemia and insulin-compensated hyperglycemia on the myo-inositol content of adult rat testes were examined. As shown in Table I, 72 hours after intravenous injection of streptozotocin (65mg/kg), rats had blood plasma glucose concentrations significantly higher ($P<.001$) than control animals which had been injected with citrate buffer in sham operations. In addition, diabetic rats gained significantly less ($P<.05$) weight during the 3 day experiment than did control animals. Although caloric intakes for both groups were not measured, this discrepancy in weight gains was probably not due to dietary factors but was a result of dehydration caused by polyuria observed in the streptozotocin injected rats.

Plasma glucose levels in diabetic rats decreased to a final value of 95.4 mg%, significantly lower ($P<.001$) than control plasma glucose concentration, 4 hours after insulin injection. Testes glucose was significantly higher ($P<.02$) and glucose-6-phosphate levels were lower ($P<.01$) than control values in diabetic and insulin treated diabetic animals respectively.

Testes myo-inositol content in diabetic animals was approximately 2.7 fold greater than control levels. Insulin treatment of diabetic rats for 4 hours lowered testes myo-inositol levels; however, the myo-inositol content remained

TABLE I

myo-Inositol Content of Testes and Plasma
of Normal, Diabetic, and Insulin Injected Diabetic Rats[a]

Metabolite	Control	Diabetic	Insulin Injected Diabetic
Plasma Glucose (mg%)	181.5 ± 14.0[3]	498.8 ± 77.1[3]	95.4 ± 5.5[3]
Plasma Glucose prior to insulin (mg%)	---	---	478.1 ± 36.0
Testes Glucose (umoles/g)	1.14 ± 0.19[2]	2.19 ± 0.67[3]	0.313 ± 0.079[3]
Testes G-6-P (nmoles/g)	30.9 ± 3.3[1]	42.0 ± 3.5[3]	23.4 ± 1.0[1]
Testes myo-Inositol (umoles/g)	1.14 ± 0.19[3]	2.19 ± 0.25[1]	1.57 ± 0.22[1]
Plasma myo-Inositol (uM)	28.0 ± 5.5[1]	16.4 ± 1.0[1]	24.4 ± 4.1

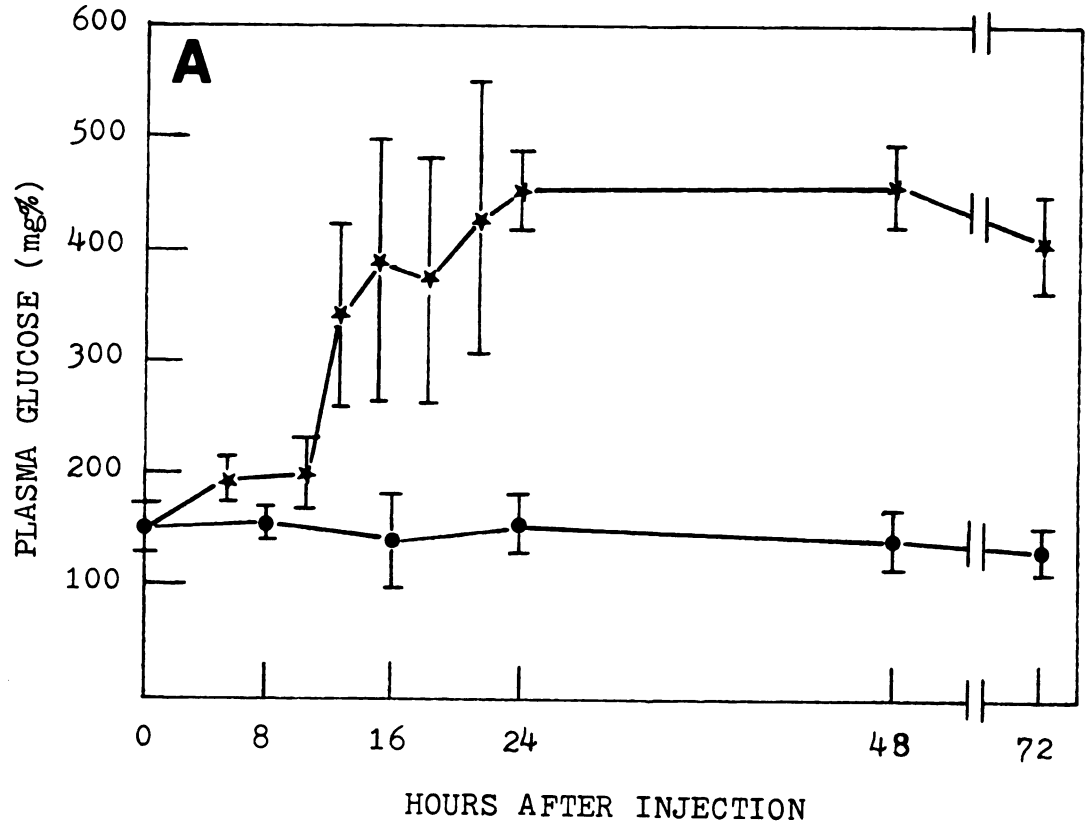
[a]All rats initially weighed 210-220 g. Streptozotocin and insulin injections were done as described in the Methods section. Values represent the mean ± standard deviation of 4 controls, 5 diabetics, or 4 insulin injected diabetics. Statistics: superscripts in each column refer to the following comparisons: control column---control vs. diabetic; diabetic column---diabetic vs. insulin injected; insulin injected column---insulin injected vs. control. The following numbers refer to the level of significance using the two-tailed t-test: [1]$P<.01$, [2]$P<.02$, [3]$P<.001$

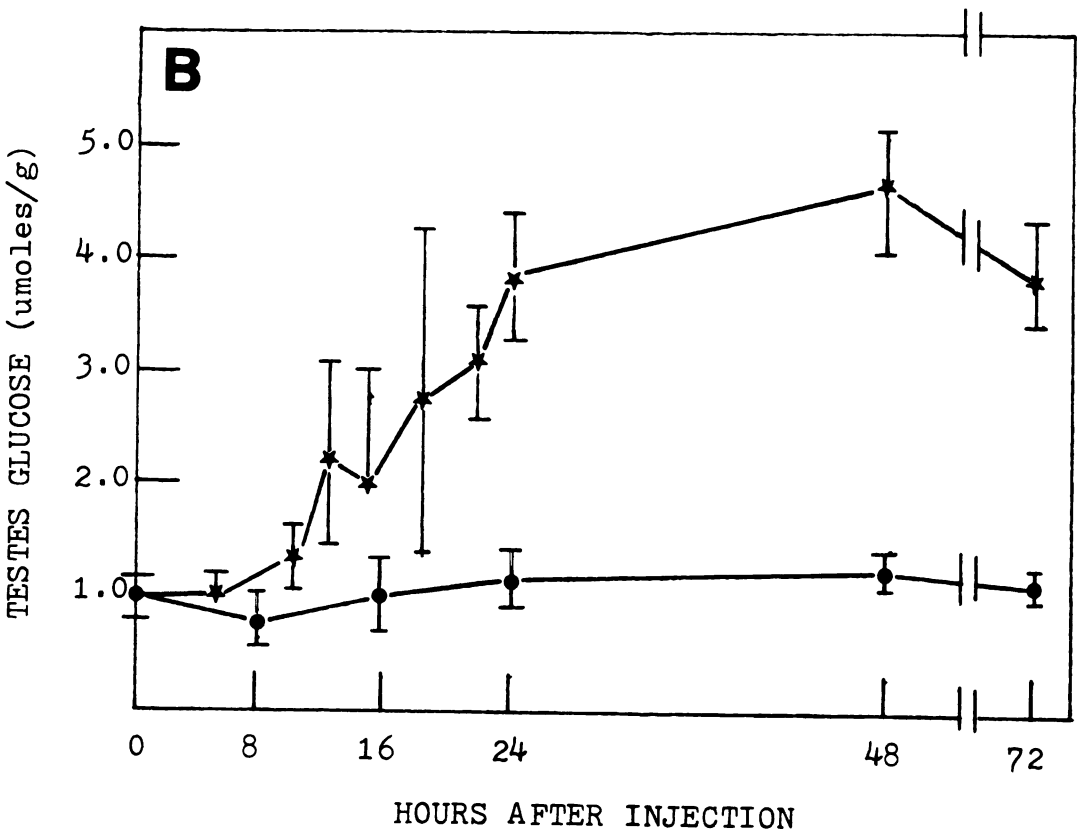
greater than control values (approximately 1.9 fold). Plasma myo-inositol levels in streptozotocin diabetic rats were unexpectedly lower ($P<.01$) than levels observed in control or insulin injected diabetic animals.

Kinetics of Streptozotocin Induced Diabetes and Effects on Testis and Plasma myo-Inositol Levels. The time course of streptozotocin induced diabetes in male rats, and its effect on testes glucose, glucose-6-phosphate, myo-inositol, and plasma myo-inositol levels were determined. As depicted in Figure 1A, plasma glucose levels rose sharply from a value of 200 mg% at 10 hours after injection of streptozotocin to 340 mg% at 12 hours. Plasma glucose continued to rise steadily after this point and plateaued 24 hours after injection at a value of 454 mg%, approximately 3 fold higher than control levels. Hyperglycemia was maintained at this level in streptozotocin injected rats for at least an additional 48 hours. Plasma glucose concentrations in control rats did not significantly deviate from approximately 150 mg% throughout the 72 hour experiment.

Testes glucose levels (Figure 1B) were observed to generally parallel plasma glucose concentrations in both control and diabetic rats. Control rats had a testes glucose content of approximately 1.0 umole/g throughout the experiment. Streptozotocin injected animals displayed a gradual increase in testis glucose 10 hours after injection which continued until approximately 24 hours after injection, reaching a final level of 3.91 umoles/g or 4 fold greater than control values. This hyperglycemia observed in the

Figure 1: Effects of Streptozotocin Injection on A) Plasma Glucose Concentration and B) Testes Glucose Content in Adult Male Rats. All rats were injected (i.v.) with streptozotocin as described in the Methods section between 9:00 and 11:00 a.m. At the indicated time after injection, rats were sacrificed and testes blood samples were collected. Each point represents the mean ± standard deviation of 4 and 3 animals for streptozotocin injected—★, and control—● rats respectively.


A
600
500
400
300
200
100
PLASMA GLUCOSE (mg%)
0
8
16
24
48
72
HOURS AFTER INJECTION


B
5.0
4.0
3.0
2.0
1.0
TESTES GLUCOSE (umoles/g)
0
8
16
24
48
72
HOURS AFTER INJECTION

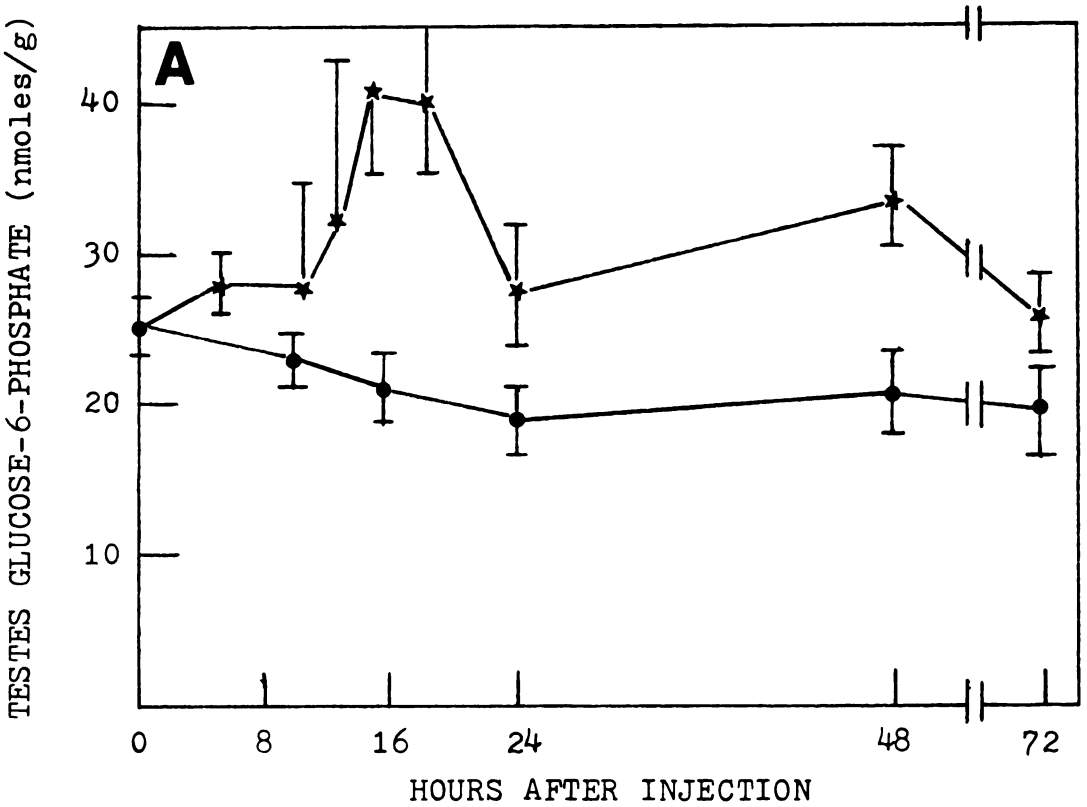
testes 24 hours after injection was maintained at nearly the same level for at least 48 additional hours.

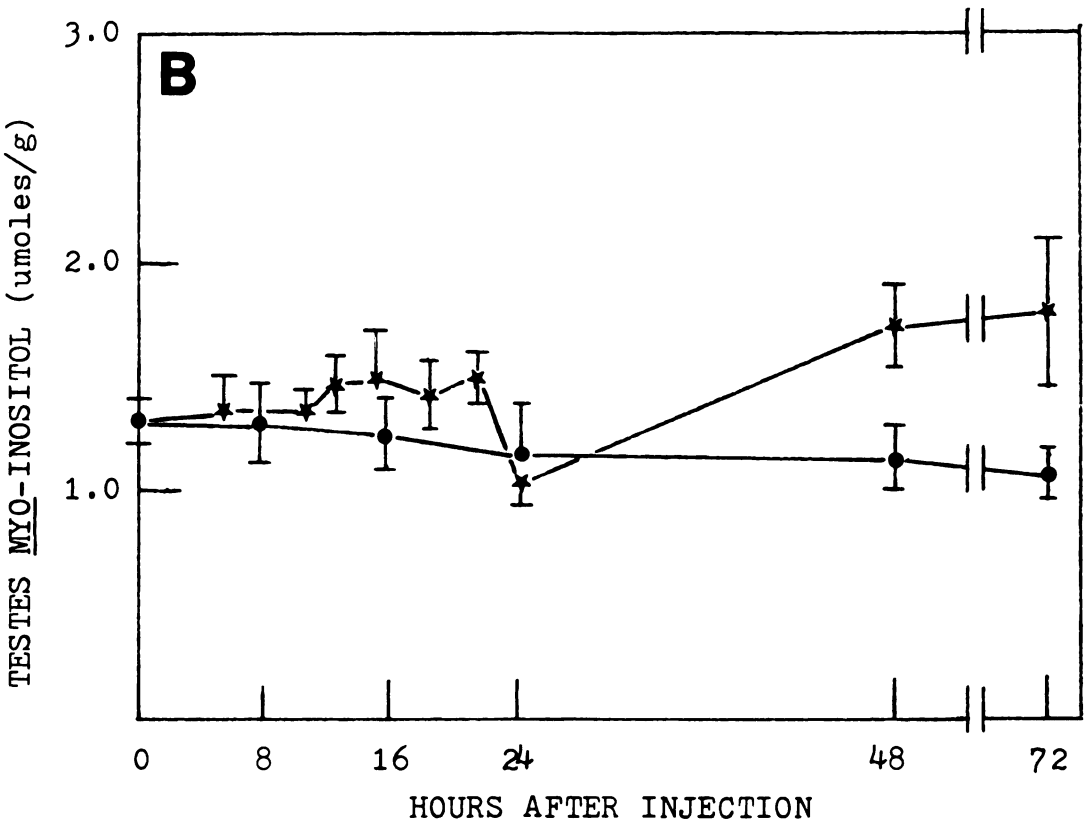
The glucose-6-phosphate content of testes from control and streptozotocin injected rats as a function of time after injection is presented in Figure 2A. Glucose-6-phosphate levels increased sharply at 12 hours after injection of streptozotocin or approximately 2 hours following the initial elevation of testes glucose levels. Glucose-6-phosphate concentration in diabetic animals plateaued between 16 and 18 hours after streptozotocin injection to a value of 40 nmoles/g. After this plateau, glucose-6-phosphate in testes decreased within 6 hours to a steady state level of approximately 30 nmoles/g or 1.5 fold greater than control values observed throughout the experiment.

The testes myo-inositol levels observed in the streptozotocin injected and control rats throughout the 72 hour interval are shown in Figure 2B. No significant differences were noted between the myo-inositol contents of the two groups during the first 24 hours. However, myo-inositol levels increased slowly at 24 hours after injection to levels of 1.762 ± 0.16 and 1.795 ± 0.40 umoles/g after 48 and 72 hours respectively. Thus, myo-inositol levels in the testes were not observed to increase until 12 and 14 hours after elevation of testes glucose-6-phosphate and testes glucose in streptozotocin injected rats.

In Figure 3 the profile of plasma myo-inositol concentrations in control and streptozotocin injected rats during the 72 hour interval following streptozotocin in-

Figure 2: Effect of Streptozotocin Injection on A) Testes Glucose-6-Phosphate Levels and B) Testes myo-Inositol Content. Conditions were as described in the Methods section and in the legend of Figure 1. Each point represents the mean ± standard deviation of 4 and 3 determinations for streptozotocin injected—✱, and control—● rats respectively.


A
TESTES GLUCOSE-6-PHOSPHATE (nmoles/g)
40
30
20
10
0
8
16
24
48
72
HOURS AFTER INJECTION


B
TESTES MYO-INOSITOL (umoles/g)
3.0
2.0
1.0
0
8
16
24
48
72
HOURS AFTER INJECTION

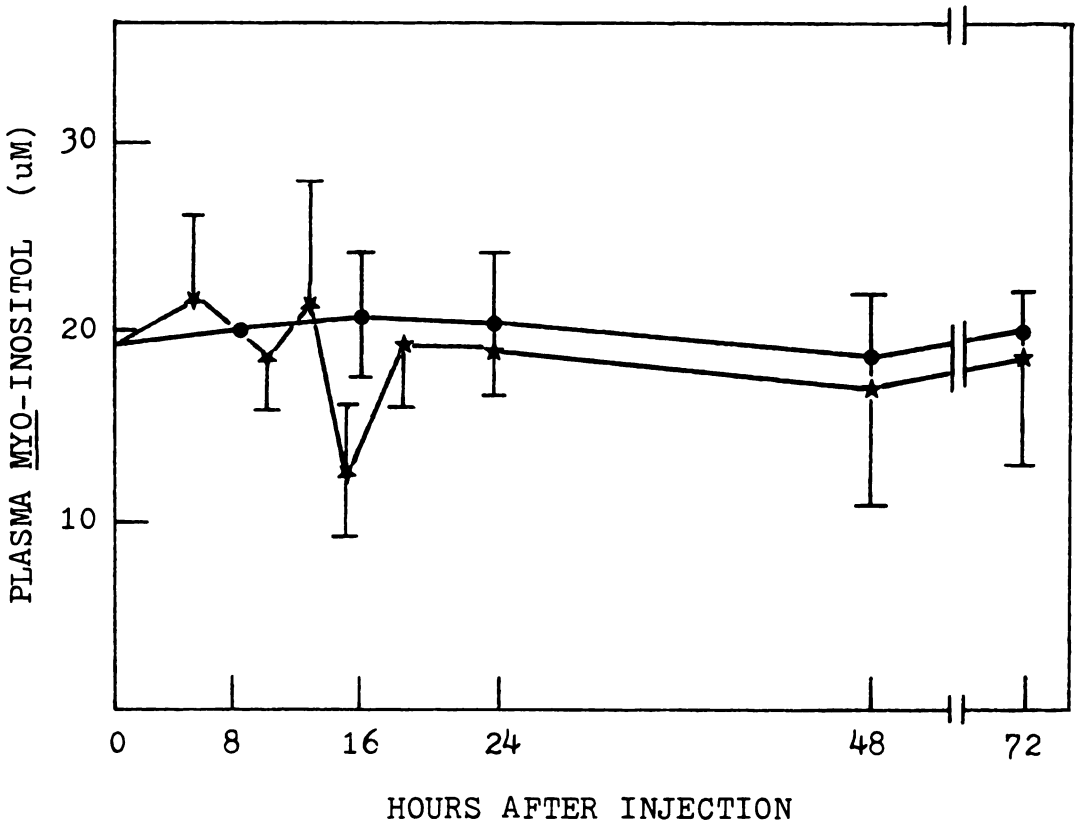


Figure 3: Effect of Streptozotocin Injection on Plasma myo-Inositol Concentration. Conditions were as described in Figure 1. Each point represents the mean ± standard deviation of 4 streptozotocin injected-★, or 3 control-● animals.

jection is shown. No significant differences were observed between the two groups. In addition plasma inositol levels did not show any diurnal variations but remained relatively constant at approximately 20 uM during the 3 day interval.

Although not shown in Figure 3, testes citrate levels were measured 24, 48, and 72 hours after injection of both diabetic and control animals. No significant differences were noted between the testes citrate levels of the two groups at these time points. In addition, no diurnal variations were noted; testes citrate levels remained unchanged during the experiment for both diabetic (0.218 $\pm$ 0.024 umoles/g, n = 12) and control (0.200 $\pm$ 0.011 umoles/g, n = 7) groups.

Synthase and Phosphatase Activities, myo-Inositol Content and Cytosolic Redox State of Testes from Normal, Diabetic, and Ethanol Fed Rats. The effects of streptozotocin induced diabetes as well as 40% (v/v) ethanol feeding on the activities of the myo-inositol biosynthetic enzymes in rat testes were investigated. In addition, the effects of these metabolic states on the myo-inositol, glucose, glucose-6-phosphate, and ATP contents and the cytosolic redox state of the testes were determined.

During the 72 hour experiment, control and diabetic rats consumed a comparable number of calories per day (82.2 $\pm$ 3.4 and 72.0 $\pm$ 6.9 respectively). Rats fed the same standard rat chow diet and a solution of 40% (v/v) ethanol ad libitum consumed 22.3 $\pm$ 9.0 ethanol calories/day and 55.2 $\pm$ 11.4 total calories/day, significantly less

($P<.05$) than the other experimental groups. The plasma glucose level of streptozotocin injected rats was 505.1 ± 51.9 mg%, significantly higher ($P<.01$) than levels observed in either control (177.6 ± 13.0 mg%) or ethanol fed (184.5 ± 12.9 mg%) animals. No significant differences were observed between the plasma myo-inositol concentrations of control (21.0 ± 7.2 uM), diabetic (29.5 ± 13.6 uM) and ethanol fed (17.2 ± 5.6 uM) rats.

As shown in Table II, no significant differences were observed in either the synthase or phosphatase activities in the testes of streptozotocin diabetic or 40% (v/v) ethanol fed rats compared to control animals. However, free myo-inositol and glucose-6-phosphate levels in the diabetic testes were elevated approximately 1.5 fold over control values. Also, myo-inositol levels in testes from ethanol fed rats were only 85% of control values. This reduced myo-inositol level in the ethanol fed rat was also paralleled by a comparable reduction in the glucose-6-phosphate content of the testes. Testes glucose concentrations were approximately 400% higher in diabetic and 15% lower in ethanol fed rats compared to control animals. While no significant differences were noted in the testes ATP levels between the three experimental groups, the testes cytosolic NAD^+/NADH ratio in diabetic rats was significantly lower than either control ($P<.01$) or ethanol fed ($P<.05$) rats.

Effects of Chronic Ethanol Consumption on Redox State and myo-Inositol Content of Liver and Testes. The effects of ethanol consumption on testes myo-inositol content were

Table II: [a]All rats initially weighed 200-225 g. All conditions were as described in the Methods section. Synthase activity units are expressed as nmoles of inositol-1-phosphate formed per hour at 37 degrees. Phosphatase activity units are expressed as umoles of inorganic phosphate cleaved from inositol-1-phosphate per 30 minutes at 37 degrees. All values represent the mean $\pm$ standard deviation of 4 controls, 5 diabetics, and 5 ethanol fed animals except for the synthase and phosphatase specific activities where n = 4 for all experimental groups.

[b]Statistics: Superscript numbers in each column refer to the following comparisons: control column---control vs. diabetic; diabetic vs. ethanol fed; ethanol fed column---ethanol fed vs. control. The following numbers refer to the level of significance using the two-tailed t-test:

$^{1}P<.05$, $^{2}P<.02$, $^{3}P<.01$, $^{4}P<.005$, $^{5}P<.001$

TABLE II

Specific Activities of the myo-Inositol Biosynthetic Enzymes, myo-Inositol and Selected Metabolite Levels, and the Cytosol Redox State of Testes from Normal, Diabetic, and Ethanol Fed Rats[a]

Parameter	Control[b]	Streptozotocin Diabetic[b]	40% (v/v) Ethanol Fed[b]
Synthase Activity (units/mg protein)	126.4 ± 13.1	121.9 ± 15.1	110.8 ± 27.4
Phosphatase Activity (units/mg protein)	0.750 ± 0.249	0.971 ± 0.199	0.841 ± 0.132
myo-Inositol (umoles/g)	1.32 ± 0.14[4]	2.09 ± 0.31[5]	0.99 ± 0.23[1]
Glucose (umoles/g)	1.25 ± 0.12[5]	5.24 ± 0.81[5]	1.02 ± 0.06[3]
Glucose-6-Phosphate (nmoles/g)	22.26 ± 2.02[3]	34.49 ± 6.06[5]	18.82 ± 0.86[2]
ATP (umoles/g)	1.81 ± 0.23	1.70 ± 0.27	1.79 ± 0.27
Lactate (umoles/g)	0.963 ± 0.073[4]	1.262 ± 0.119[5]	0.720 ± 0.083[5]
Pyruvate (nmoles/g)	36.50 ± 1.61[5]	30.20 ± 0.18[5]	21.30 ± 0.23[5]
Cytosol NAD^+/NADH	272.5 ± 31.6[3]	216.4 ± 14.9[1]	270.2 ± 46.5

reinvestigated under conditions in which ethanol fed and control animals were isocalorically pair fed and ethanol feeding was extended to a two week interval. In addition the redox states of both the liver and testes were characterized. During the experiment ethanol fed rats consumed 56.7 ± 13.8 kcal/day (n = 6), ethanol calories accounting for 53.0 ± 12.8% of all calories. In comparison pair fed controls consumed 57.3 ± 11.7 kcal/day. Body weight of ethanol fed rats decreased significantly ($P<.05$ by the two-tailed paired t-test) from 343.0 ± 7.5 g to 261.0 ± 26.8 g for a net change of -82.7 ± 27.7 g. However, the body weight of pair fed control rats did not significantly change; the initial weight was 341.0 ± 7.3 g and final weight 350.1 ± 28.0 g. The differences between the final body weights of the ethanol fed and pair fed control rats was 91.0 ± 11.5 g (significant at $P<.001$ by the two-tailed paired t-test). The discrepancy was most likely due to dehydration of the ethanol fed rats.

Table III presents myo-inositol and selected metabolites and the redox states of the liver from normal and ethanol fed rats. As shown ethanol fed rats were hypoglycemic compared to control animals, having a plasma glucose concentration approximately 28% lower ($P<.01$) than control levels. No significant alterations were observed in the cytosolic or mitochondrial redox states of the liver with ethanol feeding; however, lactate and citrate content of livers from alcohol fed rats were both about 50% lower than control levels (significant at $P<.05$ abd $P<.02$ respectively).

TABLE III

myo-Inositol and Selected Metabolite Levels and Redox State of Liver from Control and Ethanol Fed Rats[a]

Metabolite	Ethanol Fed	Control	Pairs
Glucose-6-Phosphate (umoles/g)	0.312 ± 0.059	0.300 ± 0.056	6
myo-Inositol (umoles/g)	0.263 ± 0.107	0.217 ± 0.087	4
Citrate (nmoles/g)	85.54 ± 12.71[c]	176.7 ± 8.2	5
Lactate (umoles/g)	0.253 ± 0.061[d]	0.529 ± 0.107	4
Pyruvate (umoles/g)	0.047 ± 0.009	0.091 ± 0.017	4
Cytosolic $NAD^+/NADH$	1998 ± 375	1574 ± 263	4
β-Hydroxybutyrate (nmoles/g)	114.7 ± 20.8	116.4 ± 24.3	4
Acetoacetate (nmoles/g)	26.1 ± 10.9	31.9 ± 6.3	4
Mitochondrial $NAD^+/NADH$	4.67 ± 2.07	6.24 ± 3.27	4
Plasma Glucose (mg%)	99.9 ± 21.0[b]	138.3 ± 11.8	6

[a]All male rats initially weighed 330-350 g. Ethanol fed rats received a solution of 40% (v/v) ethanol and a commercial solid diet for a two week period. Control rats were pair fed to ethanol fed rats as described in the Methods section. All values represent the mean ± standard deviation for the indicated number of pairs. The data was analyzed using two-tailed paired t-tests.

[b]Significantly different from control values at $P<.01$.

[c]Significantly different from control values at $P<.02$.

[d]Significantly different from control values at $P<.05$.

Liver myo-inositol content was not different for the ethanol fed (0.263 ± 0.107 umoles/g) animals compared to pair fed controls (0.217 ± 0.087 umoles/g). In addition, liver glucose-6-phosphate levels were unchanged upon ethanol feeding and remained at approximately 0.3 umoles/g.

The cytosolic redox state of the testes (Table IV) was not altered with ethanol feeding. No decrease in testes glucose levels in ethanol fed rats was observed in spite of the previously noted hypoglycemia of these animals. In addition, glucose-6-phosphate and myo-inositol levels of the testes of ethanol fed rats remained unchanged compared to control levels for these metabolites. Also shown in Table IV, no significant differences were observed between alcohol fed and control rats with respect to testes weight, the testes to body weight ratio, and testicular sperm count.

Intracellular Hexokinase Distribution in the Testis of Normal, Diabetic, and Alcohol Intoxicated Rats. The distribution of hexokinase activity between the cytosol and mitochondria of the rat testis was investigated in normal, streptozotocin diabetic and ethanol injected rats. Table V presents the soluble hexokinase activity of rat testis as a percentage of total hexokinase activity during the different metabolic states. As shown, neither ethanol intoxication nor streptozotocin induced hyperglycemia significantly altered the intracellular distribution of hexokinase in the testis; approximately 65% of the total activity was soluble and 35% particulate.

TABLE IV

myo-Inositol and Selected Metabolite Levels, Redox State and Sperm Count of Testes from Ethanol Fed and Control Rats[a]

Parameter	Ethanol Fed	Control
Glucose-6-Phosphate (nmoles/g)	21.08 ± 3.64	22.61 ± 2.35
Glucose (umoles/g)	1.14 ± 0.26	1.07 ± 0.08
Lactate (umoles/g)	0.950 ± 0.343	0.845 ± 0.069
Pyruvate (umoles/g)	0.019 ± 0.005	0.026 ± 0.002
Cytosolic $NAD^+/NADH$	199 ± 69	275 ± 26
myo-Inositol (umoles/g)	1.22 ± 0.15	1.00 ± 0.14
Testis Weight (g)	1.72 ± 0.11	1.72 ± 0.09
Sperm/testis x 10^{-8}	1.98 ± 0.13	2.01 ± 0.16
Sperm/g x 10^{-8}	1.16 ± 0.06	1.17 ± 0.06

[a]All conditions were as described in the legend to Table III. All values represent the mean ± standard deviation of 6 pairs of animals. No statistically significant differences for any of the parameters measured were observed.

TABLE V

Effects of Metabolic State on *In Vivo* Distribution of Testis Hexokinase

Animals[a]	n	% Soluble Hexokinase Activity[b]	Plasma Glucose (mg%)
EtOH Injected	4	62.26 ± 6.05	N.D.[c]
Control	4	67.95 ± 5.74	N.D.
Diabetic	6	70.54 ± 6.02	551.1 ± 25.2
Control	6	69.75 ± 5.24	182.3 ± 12.8

[a]All rats were initially 200-225 g. Ethanol injected rats received 10% (v/v) ethanol at a dosage of 2 g/kg 25 min before sacrifice. Diabetic rats were injected with 65 mg streptozotocin/kg 72 hr before sacrifice. Hexokinase preparations and assay were as described in the Methods section. All values represent the mean ± standard deviation for the indicated number of animals.

[b]% soluble hexokinase activity = $\frac{\text{high speed supernatant activity}}{\text{whole homogenate activity}}$ x 10

[c]N.D.---not determined

Although not shown in Table V, kinetic analyses were conducted on hexokinase preparations from rat testis cytosolic and mitochondrial fractions. The K_ms for ATP in the soluble and particulate enzymes were 0.361 ± 0.030 mM (n = 2) and 0.409 ± 0.052 mM (n = 2) respectively. The K_ms for glucose were 0.066 ± 0.011 mM (n = 3) and 0.020 ± 0.007 mM (n = 3) respectively, significantly different at $P<.002$ by the two-tailed t-test.

Investigation of the Effects of Diabetes and Concurrent Elevated Testis myo-Inositol Levels on Testicular Sperm Count and Testis Tubulin Polymerization. The effects of a two week period of streptozotocin diabetes on testicular myo-inositol levels and sperm count are depicted in Table VI. Streptozotocin injected rats had plasma glucose levels approximately 5 fold greater ($P<.01$) than control animals and testes myo-inositol levels 2.5 fold ($P<.01$) in excess of control rats. Diabetic rats did not display increased plasma myo-inositol levels, however. No significant differences were observed in the number of sperm/gram testis or in the number of sperm/testis between the diabetic and control animals. Although the testicular sperm count remained unaffected during diabetes, results from this study do not preclude any changes in the viability or fertilizing capacity of the sperm. Although the average testis weight from diabetic animals was significantly lower ($P<.005$) than control animal testis weight, the ratio of testis to body weight was significantly higher ($P<.001$) for streptozotocin injected rats compared to control animals.

TABLE VI

Effects of Diabetes and Elevated Testes myo-Inositol Levels on Sperm Counts[a]

Parameter	Control	Diabetic
Plasma Glucose (mg%)	155.1 ± 15.2	766.9 ± 105.8[b]
Plasma myo-Inositol (uM)	21.21 ± 8.45	30.75 ± 13.86
Testes myo-Inositol (umoles/g)	1.64 ± 0.21	4.27 ± 0.95[b]
Sperm/g testis x 10^{-8}	1.08 ± 0.09	1.24 ± 0.08[b]
Sperm/testis x 10^{-8}	1.68 ± 0.21	1.71 ± 0.21
Testes wt/body wt x 10^{3}	5.35 ± 0.47	7.58 ± 1.28[d]
Testis wt (g)	1.52 ± 0.06	1.01 ± 0.37[c]

[a]Rats initially weighed between 175-185 g and were 6 weeks of age. Diabetic animals were injected 2 weeks prior to sacrifice with streptozotocin as previously described in the Methods section. All values represent the mean ± standard deviation of 5 control and 9 diabetic animals. Statistical analyses were performed using the two-tailed t-test.

[b]Significant at $P < .01$.

[c]Significant at $P < .005$.

[d]Significant at $P < .001$.

Therefore, no conclusive evidence was found for testicular atrophy in diabetic rats.

Presented in Table VII are data concerning the effects of hyperglycemia on total testis tubulin and testes tubulin polymerization. Rats injected with streptozotocin 84 hours before sacrifice displayed plasma glucose levels approximately 2.7 fold higher ($P<.001$) and testes myo-inositol levels 2.1 fold higher ($P<.01$) than control levels. However, no significant differences were noted between the total tubulin content or percentage of tubulin polymerized in the testes of control and diabetic rats.

TABLE VII

Effects of Diabetes and Elevated Testis myo-Inositol Levels on Testis Tubulin Content and Tubulin Polymerization[a]

Parameter	Control	Diabetic
Plasma Glucose (mg%)	185.9 ± 11.9	499.8 ± 44.0[b]
Testis myo-Inositol (umoles/g)	1.093 ± 0.103	2.327 ± 0.849[c]
Total Testis Tubulin (pmoles/mg)	3.86 ± 0.42	4.25 ± 0.32
Testis Tubulin Percent Polymerized	38.9 ± 4.1	41.4 ± 2.9

[a]Rats initially weighed between 200-225 g. Diabetic animals were injected 84 hours prior to sacrifice with streptozotocin as previously described in the Methods section. All values represent the mean ± standard deviation of 6 animals.

[b]Significantly different from controls, $P < .001$, by the two-tailed t-test.

[c]Significantly different from controls, $P < .01$, by the two-tailed t-test.

DISCUSSION

Administration of streptozotocin, a diabetogenic antibiotic from Streptomyces achromogenes which specifically and irreversibly causes a defect in the degranulation of pancreatic β-cells (41,42), resulted in hyperglycemia and subsequent elevation of testicular myo-inositol levels in the rat. These elevated myo-inositol levels were not due to increased specific activities of the biosynthetic synthase and phosphatase enzymes in the diabetic testis. In addition, testicular uptake of myo-inositol from blood is not likely to have been responsible for the increased cyclitol levels since a testis-blood barrier has been described for myo-inositol (4,5) and no significant alterations in plasma myo-inositol concentrations were noted during streptozotocin diabetes in this study. However, elevated testicular glucose and glucose-6-phosphate levels were observed to accompany increased myo-inositol content in the testis of rats 72 hours after streptozotocin injection, suggesting that in vivo elevation of glucose-6-phosphate, substrate of the synthase, led to increased myo-inositol biosynthesis. This hypothesis was strengthened by the observation that 4 hours after insulin treatment of diabetic rats glucose-6-phosphate levels decreased to normal and concurrently myo-inositol in the testis decreased toward normal levels. The failure of testicular myo-inositol levels to return completely to normal following insulin administration to diabetic rats may be attributed to the slow turnover of the cyclitol in the testis (4).

In order to establish a direct correlation between elevated glucose-6-phosphate levels and increased myo-inositol content of the testis the concentration of these metabolites were determined during the induction of streptozotocin hyperglycemia. Investigation of the kinetics of streptozotocin effects revealed that hyperglycemia was apparent between 10 and 12 hours after injection of the drug. Testis glucose closely paralleled changes in plasma glucose concentrations while testicular glucose-6-phosphate levels increased significantly 12 hours after injection. myo-Inositol content of the testis did not increase appreciably until approximately 24 hours after streptozotocin administration. The observed lag in the elevation of the testicular myo-inositol level with respect to the initial increase in the level of glucose-6-phosphate may be due to utilization of glucose-6-phosphate primarily in other pathways. Another possible explanation rests in the observation that the cytosolic NAD^+/NADH ratio of the diabetic testis is below normal. A significantly reduced cytosolic redox-state of the testis would be expected to partially inhibit the NAD^+ requiring synthase catalyzed formation of myo-inositol-1-phosphate. Thus, although elevated glucose-6-phosphate levels favor increased myo-inositol biosynthesis, a concurrent decrease in the testis cytosolic NAD^+/NADH ratio could have an antagonistic effect and temporarily inhibit the formation of the cyclitol.

The basis for a reduced NAD^+/NADH ratio in the testis cytosol during diabetes is not well understood. The changes in the redox-state may be related to increased levels of

both acetoacetate and β-hydroxybutyrate associated with an increase in β-oxidation of fatty acids, as has been proposed for the case of the diabetic liver (43). Another proposal has been that alterations of the redox-state may be due to a disturbance in the hormonal balance between insulin and glucocorticoids during diabetes (44). However, it is concluded from these studies that the most important mode for in vivo control of myo- inositol biosynthesis is regulation of the size of the cytosolic glucose-6-phosphate pool.

The in vivo regulation of testicular myo-inositol levels by glucose-6-phosphate pools was evaluated in an ethanol-fed model. Ethanol feeding of male rats was expected to result in hypoglycemia and possibly lowered testicular glucose-6-phosphate levels. In addition, a reduced testis cytosolic NAD^+/NADH ratio was expected due to the pyridine nucleotide-linked oxidation of ethanol. These metabolic conditions would be predicted to synergistically act to decrease myo-inositol biosynthesis in the testis. Although a mild hypoglycemia was established during ethanol consumption by rats, no significant decreases in testicular glucose or glucose-6-phosphate were noted. In addition, the testis and liver cytosolic NAD^+/NADH ratios were unaltered by ethanol feeding under the conditions employed, suggesting that the various systems for transporting NADH from the cytosol to the mitochondria were capable of handling the NADH formed during ethanol oxidation. However, it is more probable that ethanol was predominantly

oxidized by the $NADP^+$ linked microsomal ethanol oxidizing system of the liver (45) and therefore little change in the cytosolic $NAD^+/NADH$ ratio occurred in either the liver or testis. Testicular myo-inositol levels were also unaltered during ethanol feeding. Thus this model was inconclusive in corroborating any regulatory role of glucose-6-phosphate levels or the cytosolic $NAD^+/NADH$ ratio on in vivo testicular myo-inositol biosynthesis.

Regulation of glucose-6-phosphate pools might involve hexokinase and/or phosphofructokinase activities. Testicular citrate levels were monitored in diabetic rats in order to indirectly ascertain the effects of phosphofructokinase activity on glucose-6-phosphate levels. Citrate is a known allosteric inhibitor or phosphofructokinase, thus citrate levels should be inversely proportional to phosphfructokinase activity in vivo and inhibition of this enzyme might lead to a significantly increased glucose-6-phosphate pool. No significant differences were noted between the citrate levels of diabetic and control rats, indicating that regulation of glucose-6-phosphate levels through phosphofructokinase activity is not likely during streptozotocin diabetes.

Hexokinase equilibrium between soluble and mitochondrial forms has been proposed as a mechanism of importance for controlling glucose phosphorylation in chick brain since the kinetic parameters of the bound and soluble enzymes are significantly different (36,46). During ischemia for example, a higher percentage of total brain hexokinase

activity exists in the more active particulate fraction (46). The hexokinase distributions of rat testes were therefore measured during diabetic and alcohol-intoxicated metabolic states in order to evaluate their possible role in the regulation of the cytosolic glucose-6-phosphate pool. No significant differences in the distribution of rat testis hexokinase activity were observed in the metabolic states tested compared to the equilibrium distribution of the control animals. In addition, no differences were observed in the K_m for ATP of soluble and bound hexokinase activites from control animals. While the particulate hexokinase activity had a significantly smaller K_m for glucose compared to the soluble form in the normal testis, both enzyme forms would be expected to be saturated with glucose during most metabolic states and thus this kinetic difference is probably not important with respect to regulation of glucose-6-phosphate pools.

Normal adult mammalian testis is reported to contain predominantly Types I and II hexokinase in equal amounts (47). Testis from rat (47,48) and man(49) have also been shown to contain an unusual isoenzyme of hexokinase designated sperm type since it has been found in developing germinal cells and Sertoli cells. Thus, the testis contains a complex collection of hexokinase isoenzymes compared to the brain, for example, which contains approximately 98% Type I hexokinase(50).

Although no changes were observed in the distribution of total hexokinase activity during different metabolic

states, these results do not preclude the possibility that redistribution of specific hexokinase isoenzymes does occur. Katzen et al.(51) have shown that streptozotozin induced diabetes in rats results in the virtual dissappearance of Type II hexokinase from the supernatants of crude homogenates of heart and fat pad, while Type I hexokinase was unaffected. Total hexokinase specific activity may also be altered during metabolic states. It has been demonstrated that a 60% decrease in the hexokinase specific activity of rat heart muscle occurs during streptozotocin diabetes (51).

Testicular degeneration and decreased spermatogenesis have been reported in genetically selected rats after eight months of uncontrolled diabetes (22). In addition, ten months of a diabetic state resulted in hyperplasia of Leydig cells in these rats. Reduced sexual behavior (16), reduced accessory sex organ weights (17-19), and degenerative changes in seminiferous tubules (20-22) have also been described in diabetic rats. Hypogonadism (23,24) and germinal cell injury (25-28) have been reported during chronic alcoholism in experimental animals and man. Whether myo-inositol is involved either directly or indirectly in these fertility dysfunctions has not been elucidated. Preliminary results from this study indicate that sperm counts are unaffected by significantly elevated testicular myo-inositol levels in streptozotocin injected diabetic rats after two weeks. In addition, sperm counts were not significantly changed in rats fed 40% (v/v) ethanol for two weeks, however, myo-inositol levels were not significantly

altered in this system. Although not presented here, current work in this laboratory suggests that streptozotocin diabetic male mice are unable to impregnate normal female mice, but impotence is not displayed in these male mice.

While the exact role of myo-inositol in the testis is unknown, the cyclitol may be important in mitotic processes. Early reports by Chargaff et al. (52,53) proposed that myo-inositol counteracted the antimitogenic activity of colchicine. The basis for this antimitogenic activity may be due to the disruption of microtubules (54). Therefore, the effect of elevated testicular myo-inositol in diabetic rats on microtubule formation through tubulin polymerization in the testis was investigated. However, no significant differences were noted between the percentages of total tubulin polymerized in the testis of normal and streptozotocin diabetic rats. These results neither prove nor disprove the existence of an important role for myo-inositol in fertility and further work is required to resolve this question.

In vivo myo-inositol biosynthesis in the rat testis system has been shown to be directly and primarily regulated by the size of the glucose-6-phosphate pool during normal and diabetic metabolic states. Elevated glucose-6-phosphate in the diabetic testis appears to result simply from an increased flux of glucose into the testis and subsequent conversion to glucose-6-phosphate. Whether testis hexokinase isoenzymes become saturated with glucose or whether a specific redistribution of theses isoenzymes occurs during

diabetes which subsequently affects the glucose-6-phosphate pool remains to be determined. myo-Inositol levels in the testis increase approximately three fold after 72 hours of hyperglycemia and remained at this level for at least two weeks after injection of streptozotocin. Although elevated myo-inositol levels caused no observed adverse effects on testicular sperm counts, further research will be required to determine the effects of myo-inositol on fertility and its role in the testes and accessory sex organs.

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