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# GALACTOSE TOXICITY AND METABOLISM IN THE CHICK (GALLUS DOMESTICUS)

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GALACTOSE TOXICITY AND METABOLISM IN THE CHICK (GALLUS DOMESTICUS)

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TO JEANNE, STACEY AND FAMILY

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| NaF | Sodium fluoride |
| NaCl | Sodium chloride |
| $Na_2MoO_4$ | Sodium molybdate |
| $MgSO_4$ | Magnesium sulfate |
| $MgCl_2$ | Magnesium chloride |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| $CaCO_3$ | Calcium carbonate |
| $CaHPO_4$ | Calcium hydrogen phosphate |
| KI | Potassium iodide |
| KCl | Potassium chloride |
| KOH | Potassium hydroxide |
| $ZnCO_3$ | Zinc carbonate |
| $CuSO_4$ | Copper sulfate |
| HCl | Hydrochloric acid |
| $(NH_4)_2SO_4$ | Ammonium sulfate |
| Tris | Tris (hydroxymethyl) amino methane HCl |
| EDTA | (Ethylenedinitrilo)-tetra acetic acid |
| DEAE | Diethylaminoethyl |
| DTT | Dithiothreitol |
| P | Phosphate |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |

LIST OF ABBREVIATIONS -- Continued

| | |
|---|---|
| UTP | Uridine-5'-triphosphate |
| UDP | Uridine-5'-diphosphate |
| ATP | Adenosine-5'-triphosphate |
| ADP | Adenosine-5'-diphosphate |
| AMP | Adenosine-5'-monophosphate |
| Glc | Glucose |
| Gal | Galactose |
| OD | Optical density |
| M | Molar |
| mM | Millimolar |
| μmole | Micromole |
| μCi | Microcurie |
| Kg | Kilogram |
| g | gram |
| mg | Milligram |
| μg | Microgram |
| mg $^{o}/_{o}$ | Milligram per 100 milliliters |
| ml | Milliliter |
| rpm | Revolutions per minute |
| cm | Centimeter |
| nm | Nanometer |
| w/v | Weight to volume |
| v/v | Volume to volume |
| Galactokinase | ATP-D-galactose-1-phosphotransferase |
| Transferase | UDP glucose; α-D-galactose-1-phosphate uridyl transferase |
| Epimerase | UDP galactose-4-epimerase |

# GALACTOSE TOXICITY AND METABOLISM IN THE CHICK (GALLUS DOMESTICUS)

## CHAPTER I

## INTRODUCTION

Galactosemia is a seriously debilitating disorder of metabolism which was first described by Reuss in 1908 (1). In humans this inborn error of metabolism is brought about when the individual cannot metabolize galactose. The disorder manifests itself in vomiting, enlarged liver and spleen, bilateral cataracts, extremely slow psychomotor development, mental retardation, and a general failure to thrive. Complete remission from most of the symptoms can be expected if galactose is eliminated from the diet at an early age. The major source of galactose in an infant's diet is from the hydrolysis of lactose, which is a major constituent of milk.

Ever since galactosemia was described, researchers have endeavored to discover the metabolic origins of the disease and the compound or compounds responsible for mental retardation. Kosterlitz, in 1943,(2) first showed that galactose-fed rabbits accumulate galactose-1-P in the eye lens. He later demonstrated that galactose adapted Saccharomyces cerevisiae could ferment galactose-1-P at a rate equal to glucose-1-P (3) and postulated the metabolism of galactose-1-P

through glucose-1-P. Five years later with galactose-adapted Saccharomyces fragilis, Leloir and co-workers (4) identified galactokinase, the first enzyme in the galactosal pathway. Subsequently, he and his colleagues identified UDP glucose as a key "co-factor" (5), demonstrated the existence of UDP galactose-4-epimerase (6), and postulated a third enzyme to promote the conversion of galactose-1-P to UDP galactose (7). Kalckar, et al. identified this enzyme as galactose-1-P uridyl transferase in S. fragilis (8). During this same period of time Schwarz, et al. (9) conclusively demonstrated the accumulation of galactose-1-P in erythrocytes of galactosemic patients. Kalckar and co-workers then showed that erythrocytes and liver from galactosemic patients were devoid of galactose-1-P uridyl transferase activity and that the other two enzymes of this pathway were normal (10,11). The major metabolic pathway for the conversion of galactose to glucose-1-P is as follows:

(a) Gal + ATP $\longrightarrow$ Gal-1-P + ADP

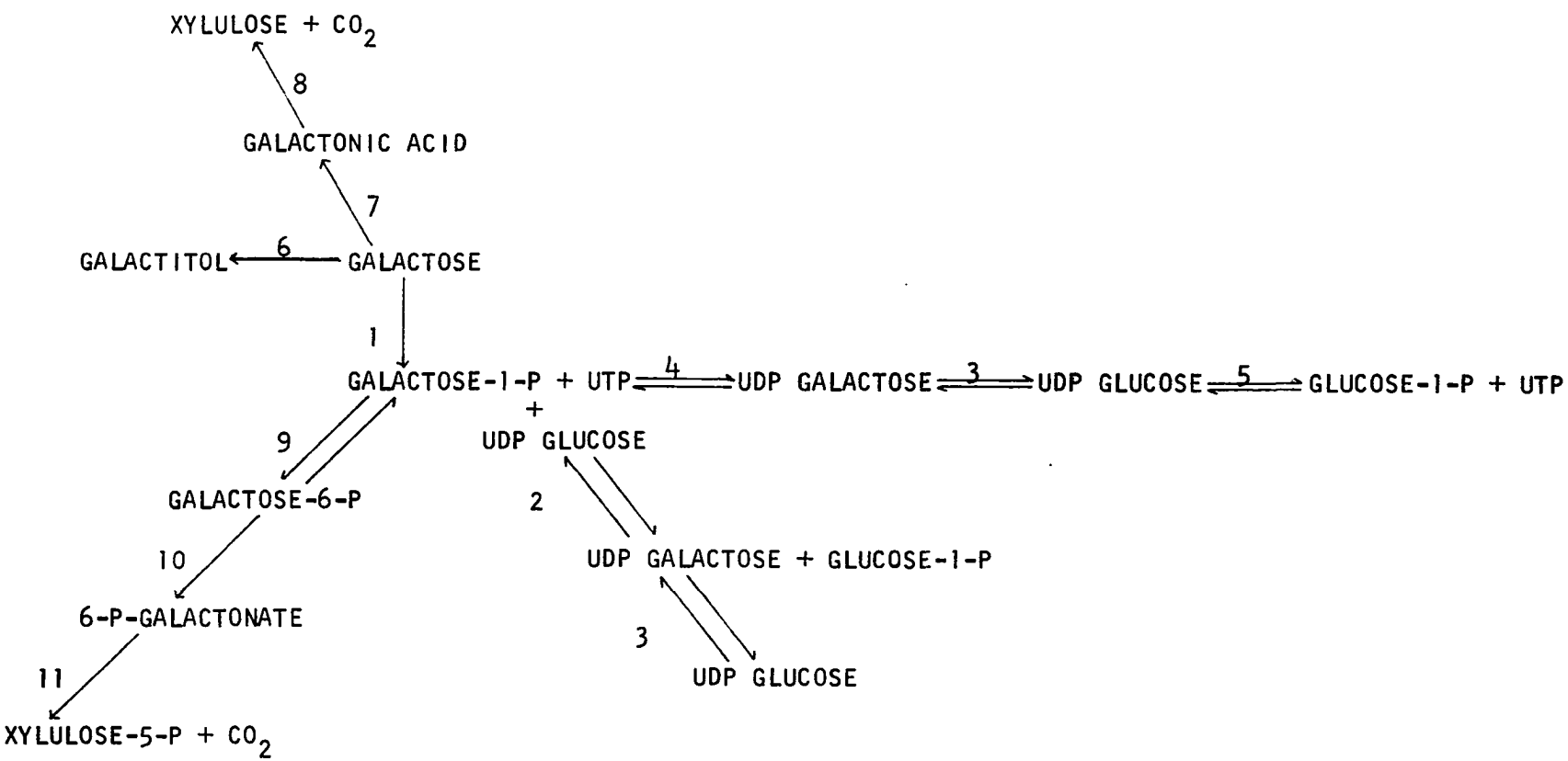
(b) Gal-1-P + UDP glc $\rightleftharpoons$ UDP gal + Glc-1-P

(c) UDP gal $\rightleftharpoons$ UDP glc

sum of b and c Gal-1-P $\rightleftharpoons$ Glc-1-P

A number of other pathways have been described which could potentially metabolize galactose (figure 1). Among these is the UDP galactose pyrophosphorylase first described by Kalckar in yeast (8). This enzyme activity was later demonstrated in mammals (12,13). At present the metabolic role of UDP galactose pyrophosphorylase is disputed as is its separate existence from UDP glucose pyrophosphorylase (14). A second pathway for the metabolism of galactose was postulated

Figure 1. The metabolism of galactose by the Leloir pathway and the postulated auxiliary pathways. The Leloir pathway enzymes are (1) galactokinase, (2) galactose-1-P uridyl transferase and (3) UDP galactose-4-epimerase. Enzymes of the phosphorylase pathway are (1) galactokinase, (4) UDP galactose pyrophosphorylase, (3) UDP galactose-4-epimerase and (5) UDP glucose pyrophosphorylase. The enzyme for direct reduction is (6) aldose reductase. Enzymes of the direct oxidative pathway are (7) galactose dehydrogenase and (8) galactonic acid dehydrogenase. Enzymes of the oxidative pathway are (1) galactokinase, (9) phosphchexomutase, (10) hexose-6-P dehydrogenase and (11) 6-phosphogalactonate dehydrogenase.


XYLULOSE + $CO_2$
8
GALACTONIC ACID
7
GALACTITOL
6
GALACTOSE
1
GALACTOSE-1-P + UTP
4
UDP GALACTOSE
3
UDP GLUCOSE
5
GLUCOSE-1-P + UTP
+
UDP GLUCOSE
9
GALACTOSE-6-P
2
UDP GALACTOSE + GLUCOSE-1-P
10
6-P-GALACTONATE
3
UDP GLUCOSE
11
XYLULOSE-5-P + $CO_2$

after finding that galactose-1-P could be converted to galactose-6-P (15) and subsequently be oxidized in the hexose monophosphate shunt (16). Galactose-6-P has also been identified in the erythrocytes of galactosemic patients (16). Finally, two other pathways were discovered; one for the direct oxidation and the other for the direct reduction of galactose. The direct reduction occurs via an aldose reductase and converts galactose to galactitol (17,18,19). This appears to be a dead-end pathway as the product accumulates in various tissues and is finally excreted via the urine. The direct oxidative pathway for galactose converts it to xylulose via several enzymatic steps (20,21,22). The evidence for this direct oxidation is currently disputed (23).

The significance of three of these auxiliary pathways of galactose metabolism in humans and in animal models is currently a subject of controversy. The fourth pathway leads to a product, galactitol, which apparently cannot be metabolized and accumulates in the tissues. To date the Leloir pathway appears to be the only major metabolic pathway for metabolizing galactose in normal individuals, but in some metabolic disorders the auxiliary pathways may be of significance in the metabolism of galactose.

Work with galactosemic individuals has shown that the most definitive test for the disorder is the absence of galactose-1-P uridyl transferase activity in their erythrocytes. It has also been shown that there are a number of variants of galactosemia. If the normal amount of transferase activity is set at 100%, the heterozygote for galactosemia has about 50% activity and the homozygote has little or

no transferase activity. In the Duarte variant, the homozygous Duarte variant has only one-half the transferase activity and the heterozygote for Duarte variant has about 75% the activity of normal individuals. Only the homozygote with no transferase activity demonstrates pathologic evidence of galactosemia. Both galactosemia and the Duarte variant are inherited as autosomal recessive traits and mating of individuals with these two distinct variants produces the distribution of transferase activities predicted by classical Mendelian genetics.

A second type of galactosemia was discovered by Gitzelmann in 1965 (24). In this type of galactosemia the galactokinase activity (first enzyme in the pathway) is not present in erythrocytes. Segal, in a recent review (25), has referred to the two types of galactosemia as transferase deficiency galactosemia and galactokinase deficiency galactosemia. A comparison of the metabolites which accumulate and the clinical symptoms which they produce is seen in the following table (Table 1). The only symptom common for both the transferase deficiency galactosemia and the galactokinase deficiency galactosemia is the development of cataracts. The formation of cataracts in these disorders appears to be a direct consequence of galactitol accumulation in the lens. The only compound which does not accumulate in both types of galactosemia is galactose-1-P. This indicates that galactose-1-P may be the compound responsible for mental retardation, renal dysfunction, and liver damage, but there is no solid evidence to date to support this hypothesis.

Chickens have been used as a model for the study of galactose toxicity since 1944 (26). Dietetically induced galactose toxicity is

TABLE 1

DISORDERS OF GALACTOSE METABOLISM

| Symptom | Galactokinase deficiency Galactosemia | Transferase deficiency Galactosemia |
|---|---|---|
| Cataracts | + | + |
| Mental Retardation | - | + |
| Liver Damage | - | + |
| Kidney Dysfunction | - | + |
| Compounds accumulating in the tissues | Galactitol, Galactose | Galactitol, Galactose, Galactose-1-P |

manifested by slow weight gain, drooping wings, tremors, seizures, and ultimate death (26,27,28,29). Pathologic studies (30) on chicks that had convulsions and were either killed or died as a result of galactose intoxication demonstrated no macroscopic changes. The only significant histologic lesions observed were in the brain. Neurons of the basal ganglia, medulla and occipital lobes showed degeneration. The degree of toxicity has been shown to differ according to sex and breed and the amount of galactose in the diet or drinking water. The concentrations of galactose (31,32) galactose-1-P (27,32,33), galactitol (31,32), and UDP galactose (34) have been shown to increase in tissues of galactose toxic chicks. Aberrations of energy metabolism (35) as well as phospholipid (35) and glycoprotein (36) metabolism, and osmolality (37,38,39) have been reported.

Sex-related differences in the mortality of newly hatched chicks on a galactose diet have been demonstrated, with more males surviving than females (29). Greater amounts of galactose-1-P have been measured in brains of galactose-fed females than in those of males (33). Moreover, females exhibit less liver and brain transferase activity than males (33,40). This sex difference is not observed in humans, as there are no differences between males and females in the severity of the clinical manifestations in galactosemia or in erythrocyte transferase activity in normal individuals (25).

Chickens have been used as an animal model for galactosemia because they react to ingestion of galactose with a general failure to thrive and a type of neurotoxicity which it is believed reflects a mechanism similar to that found in human galactosemia. The

neurotoxicity is not common to other animals used to study the toxic effects of galactose. The chicks do not appear to develop cataracts, enlarged liver and spleen or other tissue damage found in galactosemic patients. Despite the extensive studies in galactosemia, molecular mechanisms for most of the clinical manifestations have not been elucidated in prior work. The elucidation of the causes of the galactose toxicity in chicks may therefore yield information directly pertinent to clinical medicine, as well as increase our knowledge of metabolic lesions.

The purpose of this investigation is to further characterize the chicken as an animal model in the study of galactose intoxication and to define the molecular mechanism for neurotoxicity in the chick. This will be accomplished by studying a) the development of the galactose metabolizing enzyme activities in the livers of male and female chicks with increasing age; b) the characteristics of purified transferase from livers of male and female chicks; c) mortality of chicks on a galactose supplemented diet as a function of age and sex; d) concentrations of brain glucose, galactose and galactose-1-P and blood glucose and galactose in control and galactose-fed chicks as a function of age and sex; and e) the relationships between each of these parameters.

# CHAPTER II

# MATERIALS AND METHODS

## Chick and Tissue Preparation

The dietary study was designed to assess the influence of age, as well as sex, on the susceptibility of the chick to galactose toxicity. To do this the mortality study was divided into three phases, day-old chicks were in the first phase, three-week-old chicks in the second phase, and six-week-old chicks in the third phase. Within each phase the results were compared between male and female and between experimental and control.

Newly hatched male and female leghorn chicks were obtained from a local hatchery. They were fed either a control diet or a galactose supplemented diet (Table 2). The basal diet was a modification of that described by Kokatnur, et al. (41). The author omitted the p-amino benzoic acid and added powdered, non-nutritive cellulose for bulk. In the galactose diet, galactose was added at the expense of glucose so that it accounted for 18% of the total diet. To study galactose toxicity, one hundred forty chicks of each sex were started on a control diet and sixty of each sex began receiving an 18% galactose diet. In the second phase, chicks in the original control group were continued on their control diet until day 21 of life. At

TABLE 2

COMPOSITION OF CHICK DIET

| Constituent | Control g/4.4Kg | Experimental g/4.4Kg |
|---|---|---|
| Protein (Soybean) | 1412.0 g | 1412.0 g |
| Glucose | 1926.0 g | 1126.0 g |
| Galactose | - | 800.0 g |
| Methionine | 30.0 g | 30.0 g |
| Glycine | 12.0 g | 12.0 g |
| Salt Mix[a] | 210.8 g | 210.8 g |
| Choline Chloride | 8.0 g | 8.0 g |
| Vitamin Mix[b] | 1.2 g | 1.2 g |
| Corn Oil (Stripped) | 400.0 g | 400.0 g |
| Non-Nutritive Cellulose | 400.0 g | 400.0 g |

[a]Salt Mixture for Chicken Diet:

| Compound | g/20Kg |
|---|---|
| $CaCO_3$ | 433.2 g |
| $KH_2PO_4$ | 210.0 g |
| $CaHPO_4$ | 188.0 g |
| NaCl | 160.0 g |
| $MgSO_4$ | 50.0 g |
| $FeSO_4 \cdot 7H_2O$ | 6.0 g |
| $MnSO_4 \cdot H_2O$ | 4.0 g |
| $ZnCO_3$ | 2.0 g |
| $CuSO_4$ | 0.4 g |
| KI | 0.2 g |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.2 g |

[b]Vitamin Mixture for Chicken Diet:

| Vitamin | g/20Kg |
|---|---|
| Thiamine | 2.0 g |
| Niacin | 2.0 g |
| Riboflavin | 0.32 g |
| Ca Pantothenate | 0.40 g |
| Vitamin $B_{12}$ | 0.0004 g |
| Pyridoxine | 0.120 g |
| Folic Acid | 0.080 g |
| Menadione | 0.100 g |
| Vitamin $D_3$ | 0.003 g |
| $\alpha$-Tocopherol | 2.0 g |
| Biotin | 0.012 g |
| Vitamin A Acetate | 200,000 IU |

this time, they were divided into four groups: a) 60 females began receiving an 18% galactose diet: b) 60 males began receiving the same galactose diet: c) 80 females were kept on control diet; and d) 80 males were kept on control diet. For the third phase, 60 chicks of each sex were fed commerical non-medicated chick feed until the 42 day of life at which time they were divided into four groups of 30, one male and one female group began receiving the 18% galactose diet and one group of each sex was placed on control diet. In each of the three phases, chicks were fed galactose and control diets while mortality was recorded for 10 days.

To determine the concentrations of galactose and glucose in serum and brain homogenate and galactose-1-P in brain homogenate, the chicks were placed on an 18% galactose diet and control diet for the first four days in each of the three phases. In the first phase, newly hatched chicks were divided into four groups; a) 40 females on galactose diet; b) 30 females on control diet; c) 40 males on galactose diet; and d) 30 males on control diet. For the second and third phases, chicks were fed commercial non-medicated chick feed until they were 21 or 42 days of age, then they were divided into four groups of 30 chicks each. One female and one male group received galactose diet and one male and one female group began control diet. Five chicks from each group, for the first four days, of each phase were bled by heart puncture, sacrificed by decapitation, and the heads were dropped into liquid nitrogen. The whole blood was placed in stoppered test tubes. The tubes stood approximately one hour at room temperature and were then placed in the refrigerator over

night. The clotted blood was then centrifuged at 1000 x g for 20 minutes at 0°C. The supernates were then decanted and stored frozen until assayed for glucose and galactose. Galactose concentrations were determined in whole serum while glucose determinations were conducted after deproteinization of the serum by the method of Somogyi (42).

The frozen brain was dissected from the skull, weighed and homogenized in 2 volumes of 0.01 M Tris-HCl buffer, pH 7.8 containing 0.14 M KCl and 0.001 M EDTA. The homogenates were then placed in a boiling water bath for 5 minutes and subsequently centrifuged at 27,000 X g for 20 minutes. Galactose and galactose-1-P concentrations were determined in the boiled supernates. Glucose determinations were conducted on boiled brain supernates which had been further deproteinized by the method of Somogyi (42).

For the enzymatic studies, Leghorn chick embryos and newly hatched chicks were utilized. Chicks were fed commercial feed. The embryos or chicks were sacrificed at intervals from day - 5 (16 days after setting) to the 50th day of life, in groups of four of each sex. Immediately after sacrifice, the liver was rapidly removed and homogenized in a glass-teflon homogenizer in 2 volumes of 0.01 M Tris-HCl buffer, pH 7.8, containing 0.14 M KCl and 0.001 M EDTA. The homogenate was centrifuged at 27,000 X g for 30 minutes at 0°C. Centrifugation was repeated on the supernatant fraction at 100,000 X g for 1 hour at 0°C. The resulting supernate was then assayed for galactokinase, transferase and epimerase activity. Protein was

determined by the method of Lowry et al. (43).

Glucose, Galactose and Galactose-1-P Determinations

Glucose concentrations in serum and brain homogenate were measured using glucose oxidase, semi-micro method (Worthington Biochemicals).

Determinations of galactose in the serum and brain homogenates were made by end point assays using galactose dehydrogenase followed spectrophotometrically by the reduction of NAD at 340 nm. Formation of μmoles of product were based on a molar extinction coefficient of $6.22 \times 10^3$ for NADH at 340 nm. The reaction mixture contained 0.035 units of galactose dehydrogenase (Sigma), 1.0 μmole NAD, an appropriate amount of serum or brain extract, and 0.1 M glycine buffer pH 8.7 in a total volume of 0.5 ml.

Galactose-1-P concentrations in brain homogenates were measured spectrophotometrically by the reduction of NADP at 340 nm in a reaction coupled with purified bovine transferase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The reaction mixture contained 0.005 units of purified bovine transferase; 0.4 μmole of NADP, $MgCl_2$ and UDP glucose; 0.2 μmole DTT; 0.02 IU glucose-6-phosphate dehydrogenase; 5μg crystalline phosphoglucomutase; and appropriate amounts of brain homogenate in 0.1 M glycine buffer pH 8.7 in a total volume of 0.5 ml. The concentration of galactose-1-P is based on a molar extinction coefficient of $6.22 \times 10^3$ for NADPH at 340 nm.

## Enzyme Assays

Galactokinase activity (ATP-D-galactose-1-phosphotransferase, EC 2.7.1.6), was measured by the incorporation of radioactive galactose-1-$^{14}$C into galactose-1-phosphate. The radioactive galactose-1-P formed in the reaction was trapped on DEAE cellulose paper and counted in a scintillation counter after the method of Sherman (44). The standard assay mixture included 1.0 μmole of galactose containing 0.0225 μCi of galactose-1-$^{14}$C, 2 μmoles of ATP, $MgCl_2$ and NaF, and 0.2 ml of homogenate in 0.1 M Tris-HCl buffer pH 7.8 to a total volume of 0.4 ml. A unit is defined as that amount of enzyme which catalyzes the appearance of 1.0 μmole of product per minute at 30°C.

Transferase activity (UDP glucose; α-D-galactose-1-phosphate uridyl transferase, EC 2.7.7.12) was measured by the rate of formation of glucose-1-phosphate. It was followed spectrophotometrically by the reduction of NADP at 340 nm in a coupled reaction with phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase (45). The reaction mixture contained 0.2 μmole DTT; 0.4 μmole of NADP, UDP glucose, galactose-1-P and $MgCl_2$; 5 μg of crystalline phosphoglucomutase, 2 units of 6-phosphogluconic acid dehydrogenase, 0.02 IU of glucose-6-phosphate dehydrogenase, and an appropriate amount of homogenate in 0.01 M glycine buffer pH 8.7 to a total volume of 0.5 ml.

One unit is defined as the amount of enzyme required to catalyze the formation of 1 μmole of product per minute at 30°C. Formation of 1 μmole of product is based on the molar extinction coefficient of $6.22 \times 10^3$ for NADPH at 340 nm.

Epimerase (UDP galactose-4-epimerase, EC 5.1.3.2) was assayed by the rate of formation of UDP glucose in a coupled reaction with UDP glucose dehydrogenase (46). The reaction was followed by the reduction of NAD at 340 nm. The reaction mixture contained: 0.3 μmole UDP galactose, 0.8 μmole of NAD, UDP glucose dehydrogenase and appropriate amounts of homogenate in 0.1 M glycine buffer pH 8.7 to a final volume of 0.5 ml. Formation of 1 μmole of product is based on the molar extinction coefficient of $6.22 \times 10^3$ for reduced NAD at 340 nm. One unit is defined as the amount of enzyme necessary to catalyze the formation of 1 μmole of product per minute at 30°C.

Spectrophotometric measurements were made on either an automated Gilford 240-242 recording spectrophotometer or a Beckman DU-Gilford Automated recording spectrophotometer.

## Molecular Weight Estimation by Gel Filtration

The molecular weight of the enzymes was determined by gel filtration using Sephadex G-200 by the method of Andrews (47). The gel was first suspended in 0.05 M Tris-HCl buffer containing 0.1 M KCl at pH 7.5. It was subsequently washed and equilibrated with 0.01 M phosphate buffer containing 0.001 M EDTA and 0.01 M 2-Mercaptoethanol adjusted to pH 7.0. To calibrate the column (3 X 56 cm) the following standards were applied: Catalase (Sigma-Beef liver, M.W. = 240,000), alcohol dehydrogenase (Worthington-Yeast; M.W. = 151,000), hexokinase (Sigma-type C-300; M.W. = 96,000, lactic dehydrogenase (Sigma-type III; M.W. = 142,000), alcohol dehydrogenase (Worthington-horse liver; M.W. = 80,000), and

peroxidase (Sigma-type IV; M.W. = 40,000). Catalase was assayed by the method of Beers and Sizer (48), both alcohol dehydrogenases by the method of Vallee and Hoch (49), lactic dehydrogenase assayed by the method of Kaloustian, et al. (50), the activity of hexokinase was followed by the method of Bailey and Webb (51), and horseradish peroxidase was assayed by the method of Maehly (52). All of the standards listed above and either purified female chick liver transferase or male chick liver transferase were applied to the column in a total volume of 0.5 ml which was eluted in 2.1 ml fractions. The molecular weight of transferase was estimated by comparing its elution volume with the elution volumes of known proteins plotted as a function of the log of their molecular weights.

Molecular Weight Estimation by Sucrose Gradient Centrifugation

The molecular weight of the transferases was also estimated by sucrose gradient centrifugation. A 4.6 ml linear 5-20% sucrose gradient was prepared in 0.04 M phosphate buffer pH 7.0 containing 0.001 M EDTA and 0.01 M 2-Mercaptoethanol. The 20% sucrose solution also contained Bromophenol blue (0.3 mg/ml). 2.2 ml of the 20% and 2.4 ml of the 5% sucrose solution were mixed and stored 2-4 hours before use in cellulose nitrate tubes. In each of six tubes, either male or female transferase and four standards; yeast alcohol dehydrogenase, lactic dehydrogenase, hexokinase and peroxidase in 0.05 ml were layered on top of the gradient. In another six tubes, either male or female transferase and four standards; horse liver alcohol dehydrogenase, lactic dehydrogenase, hexokinase and peroxidase in 0.05 ml were applied to the gradient. The tubes were placed in a

Ti 50.1 rotor and spun for 12 hours at 40,000 rpm in a model L2-65 Beckman-Spinco refrigerated centrifuge.

The mathematical assumptions of Martin and Ames were used in estimating the molecular weights of each of the transferases. Martin and Ames (53) demonstrated that the migration of proteins through a sucrose gradient was linear with time. This allows one to calculate the sedimentation coefficient in unknown proteins using the sedimentation coefficient of similarly shaped, known proteins according to the following proportionality:

$$\frac{S_1}{S_2} = \frac{d_1}{d_2}$$

where $S_1$ and $S_2$ represent the sedimentation coefficients and $d_1$ and $d_2$ represent the distances migrated from the top of the gradient. Once the sedimentation coefficient is known for the experimental protein the molecular weight can be estimated by comparison with the molecular weight of the known proteins by the following equation:

$$\frac{S_1}{S_2} = \frac{(Mw_1)\ 2/3}{(Mw_2)\ 2/3}$$

where $S_1$ and $S_2$ represent the sedimentation coefficients of the known and experimental protein and $Mw_1$ and $Mw_2$ represent the molecular weights of the standard and experimental molecules.

Kinetic Parameters, pH Optimum, and Temperature Sensitivity

Kinetic properties were determined for the purified liver transferases from male and female chicks. All assays were performed four times for each of the concentrations of galactose-1-P and UDP glucose used. The Michaelis constant (Km) and maximal velocity (Vmax) of the enzyme reaction for both galactose-1-P and UDP glucose were determined from Lineweaver-Burk plots for each of the purified transferases.

The pH optima were determined by incubating each of the purified enzymes in 0.1 M glycine buffers between pH 6 and pH 10. A two step assay was employed for determination of transferase activity in these studies, thus, eliminating the effect of pH on the coupling enzymes. Purified chick liver transferases were incubated in 0.1 M glycine buffer pH 6-10 containing 0.4 μmole UDP glucose and galactose-1-P and 0.2 μmole DTT at 30°C. After ten minutes incubation, the reaction was stopped by placing the tubes in a boiling water bath for 5 minutes. The amount of glucose-1-P was measured by adding NADP, $MgCl_2$, phosphoglucomutase and glucose-6-P dehydrogenase at pH 8.7 and observing the total change in optical density at 340 nm.

Temperature sensitivities of the purified enzymes were determined by incubating the enzymes in 1% bovine serum albumin solutions for 5 minutes at temperatures from 0°C to 70°C. The enzyme solutions were then cooled to 0°C for 5 minutes and the activity was determined at 30°C.

## Chemicals

All chemicals used in this research project were of reagent grade. The galactose-1-$^{14}$C was obtained from New England Nuclear. Phosphoglucomutase and glucose-6-phosphate dehydrogenase were purchased from Calbiochem. Horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase and glucose oxidase (glucostat) were obtained from Worthington Biochemical Corporation. The following enzymes were purchased from Sigma Chemical Company: 6-phosphogluconic acid dehydrogenase, hexokinase, lactic dehydrogenase, catalase, and peroxidase. UDP glucose dehydrogenase and galactose-1-P uridyl transferase were purified from calf liver by the methods of Strominger, et al. (54) and Mayes and Hansen (45).

# CHAPTER III

# RESULTS

## Mortality Studies

To study galactose toxicity, chicks were fed an 18% galactose diet. This study was carried out in three phases to determine the influence of sex and age during the consumption of galactose. Markedly different mortality patterns emerged for males and females. There were also significantly large differences between the three age groups, newly hatched, 21-day-old, and 42-day-old chicks (figures 2 & 3). The newly hatched chicks had the highest mortality during the ten days they were on galactose supplemented diet. Mortality at the end of 10 days was 97% for female chicks. Males showed sickness more slowly and mortality was only 50% at the end of 10 days. To determine the effect of age, another group of male and female chicks were placed on galactose diet at 21 days of age. Percent mortality was again determined for 10 days for this group. As in the first phase, females showed greater percent mortality than did males, but to a smaller extent. Twenty-one-day-old female chicks had 38% mortality and males of the same age had only 20% mortality at the end of 10 days. After the 3-week-old chicks were on diet for six days mortality leveled off. This indicates that some of the chicks seem to be more resistant to the toxic effects of

Figure 2. Mortality of male (——) and female (----) chicks during the first 10 days of life on an 18% galactose diet.

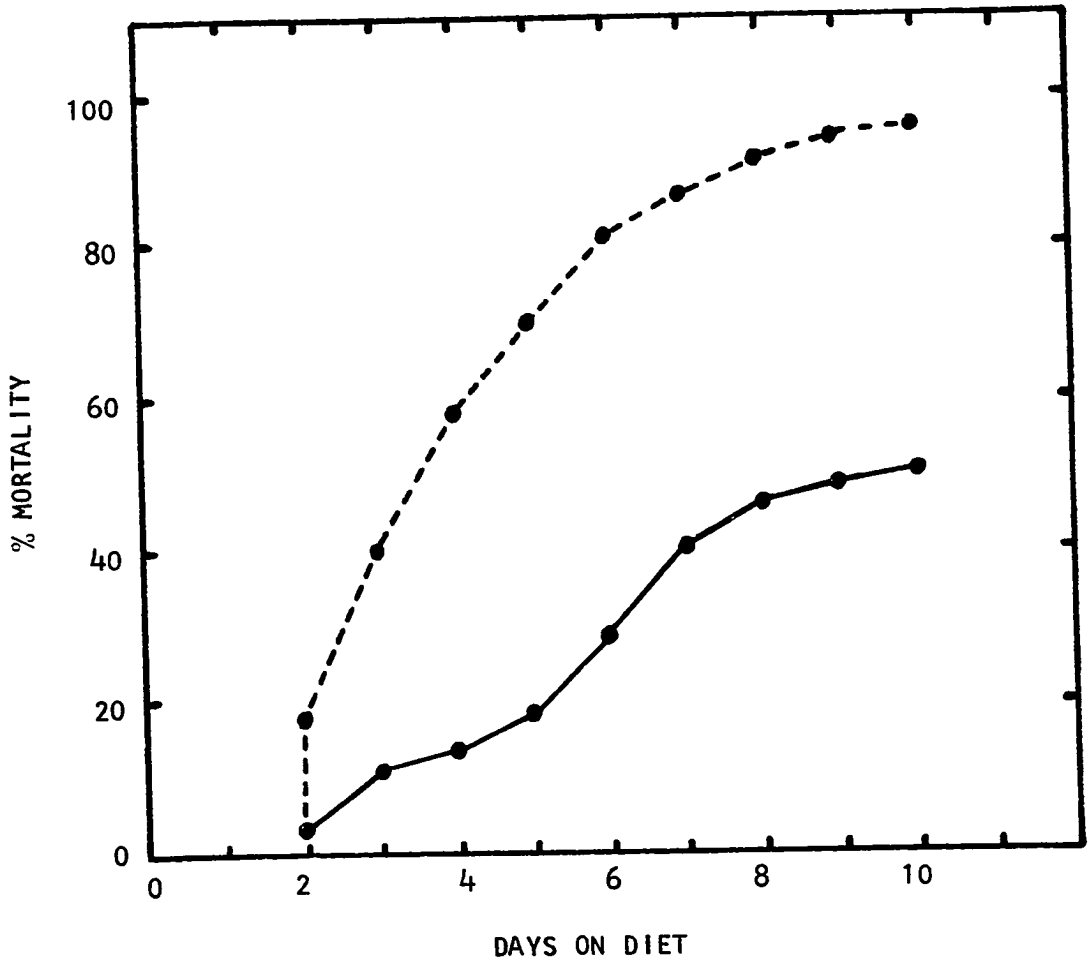
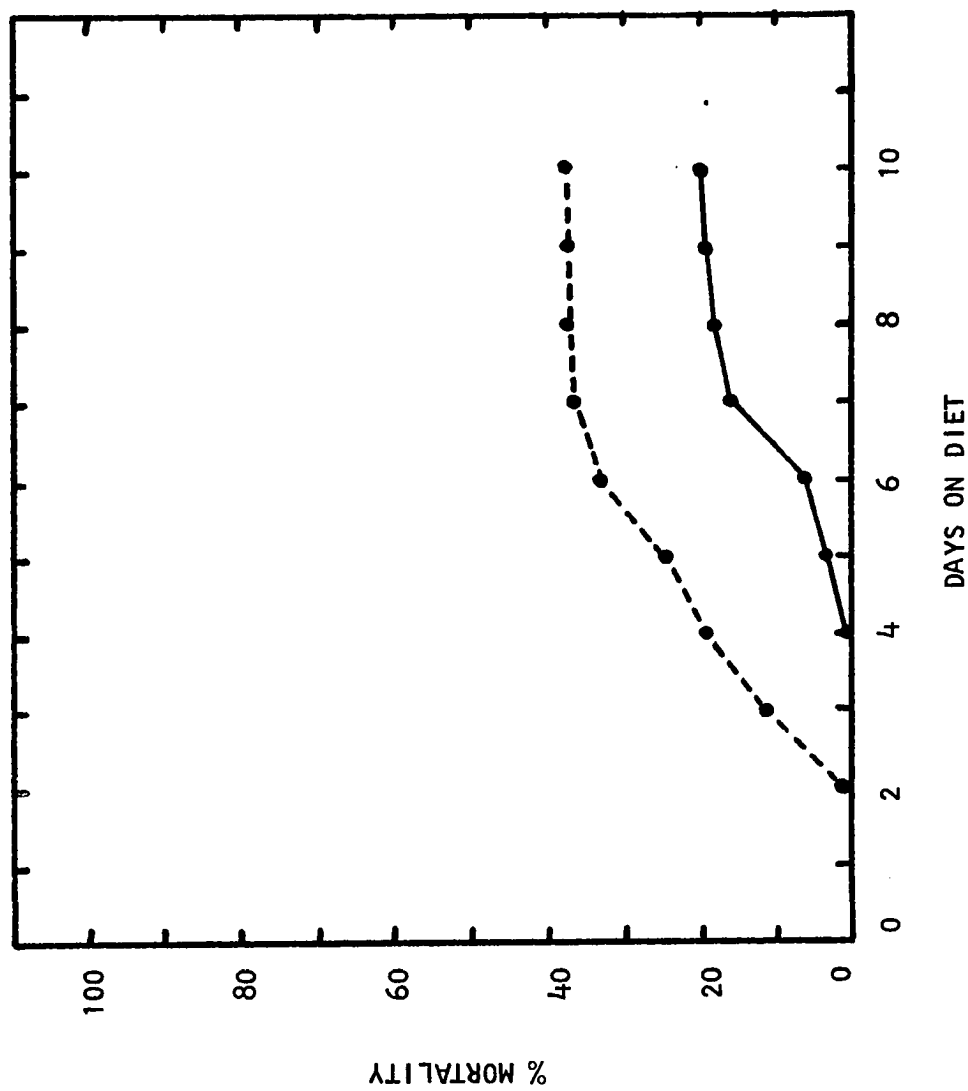

100
80
60
40
20
0
% MORTALITY
0
2
4
6
8
10
DAYS ON DIET

Figure 3. Mortality of male (———) and female (– – – ) chicks on the 18% galactose diet from the 21st to the 31st day of life.


% MORTALITY
0
20
40
60
80
100
0
2
4
6
8
10
DAYS ON DIET

galactose supplemented diet. In the third phase, chicks were fed commercial feed until they were 42 days old. At this time they were placed on the 18% galactose supplemented diet for 10 days. During this period there was no mortality for either males or females. In the control groups for each of the three phases mortality never exceeded 5%. The conclusion from this series of experiments was that both male and female chicks tolerated an 18% galactose diet much better with increasing age and the day-old and 21-day old male chicks had a better survival rate than the females of the same age.

The results from the chick mortality study and the general clinical observations could be explained by an increase in galactose metabolism with increasing age. The chick mortality study also suggested that at six weeks of age the female chick could tolerate galactose as well as males. The next experiment of this project was to determine if there were any changes in the activities of the galactose metabolizing enzymes with age and if the activities of these enzymes were similar in male and female chicks at seven weeks of age.

To accomplish this, chicks were placed on a control diet and the activities of galactokinase, transferase, and epimerase were determined in the livers of chicks from five days before hatching through fifty days of age (Table 3). Each of the three enzymes showed peak activity on or near the day of hatching (figures 4 - 6). The activities of the galactose metabolizing enzymes declined from hatching day to the second day of life where they leveled off and remained relatively constant through the fiftieth day of life. No consistent differences were apparent between male and female chicks for either

Table 3

ACTIVITIES OF THE GALACTOSE ENZYMES IN THE LIVERS OF CHICKS AT VARIOUS AGES

| Age | Galactokinase activity (Units x $10^3$/mg protein)[a] | | Transferase activity (units x $10^3$/mg protein)[a] | | Epimerase activity (units x $10^3$/mg protein)[a] | |
|---|---|---|---|---|---|---|
| | Female | Male | Female | Male | Female | Male |
| -5 | 3.28 ± .18 | 4.41 ± .83 | 1.08 ± .08 | 1.94 ± .14[b] | 2.08 ± .12 | 2.23 ± .37 |
| -3 | 3.67 ± .74 | 3.83 ± .45 | 1.51 ± .10 | 2.94 ± .17[b] | 2.07 ± .35 | 2.55 ± .17 |
| 0 | 5.67 ± .50 | 6.36 ± .70 | 2.14 ± .14 | 4.14 ± .23[c] | 4.05 ± .20 | 4.29 ± .15 |
| 2 | 3.99 ± .64 | 3.76 ± .38 | 1.54 ± .09 | 2.80 ± .16[b] | 2.00 ± .17 | 2.12 ± .10 |
| 7 | 5.03 ± .33 | 4.13 ± .61 | 1.71 ± .05 | 2.68 ± .07[b] | 1.76 ± .05 | 1.70 ± .12 |
| 14 | 3.87 ± .18 | 4.44 ± .47 | 1.41 ± .12 | 2.62 ± .27[c] | 1.57 ± .10 | 1.52 ± .16 |
| 21 | 4.73 ± .44 | 3.66 ± .21 | 1.34 ± .12 | 2.97 ± .20[b] | 1.43 ± .18 | 1.48 ± .10 |
| 28 | 4.70 ± .26 | 5.15 ± .59 | 1.57 ± .21 | 2.54 ± .14[d] | 1.50 ± .56 | 1.63 ± .33 |
| 35 | 4.02 ± .46 | 4.60 ± .05 | 1.41 ± .12 | 2.45 ± .10[b] | 1.06 ± .06 | 1.27 ± .12 |
| 42 | 4.93 ± .14 | 4.55 ± .62 | 1.37 ± .11 | 2.09 ± .19[c] | 0.81 ± .14 | 0.79 ± .08 |
| 49 | 3.51 ± .53 | 3.62 ± .55 | 1.93 ± .14 | 2.79 ± .12[c] | 0.84 ± .13 | 1.04 ± .47 |

[a] A unit is defined as the amount of enzyme needed to convert 1 µmole of substrate to product per minute at 30°C. Each value represents the mean ± SE of determinations on the livers from four animals.

[b] $p<0.001$. Significance was determined by the student T test for non-paired variants between liver transferase activities in male and female chicks.

[c] $p< 0.005$.

[d] $p< 0.01$.

Figure 4. Galactokinase activity in livers of male (——) and female (– – –) chicks. A unit of activity is defined as the amount of galactokinase needed to form 1 μmole of galactose-1-P per minute at 30° C. Each point represents the mean of determinations on the livers of four animals.

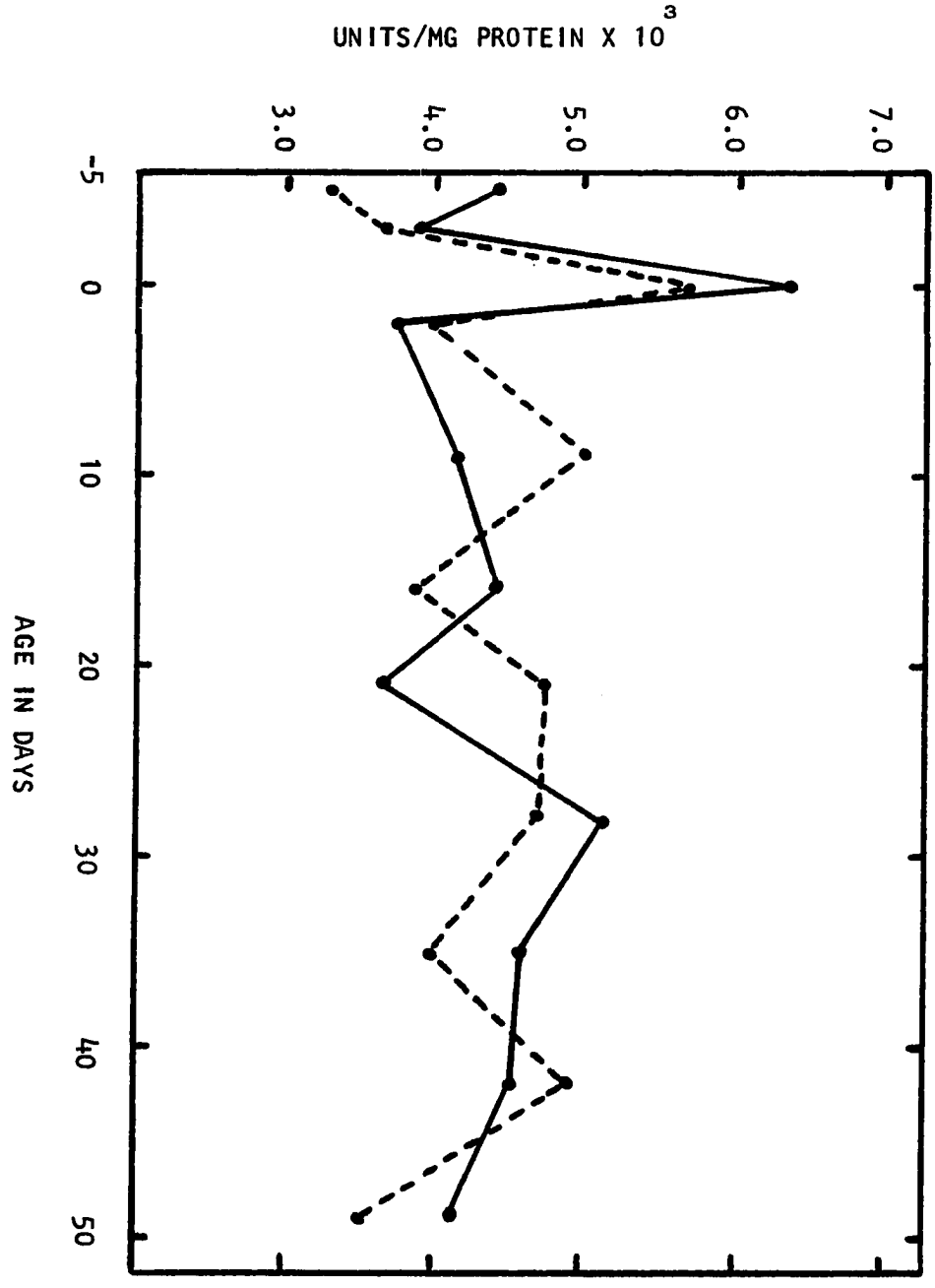

UNITS/MG PROTEIN X 10³
3.0
4.0
5.0
6.0
7.0
-5
0
10
20
30
40
50
AGE IN DAYS

Figure 5. Galactose-1-P uridyl transferase activity in livers of male (———) and female (– – – –) chicks. A unit of activity is defined as the amount of transferase needed to form 1 μmole of glucose-1-P per minute at 30° C. Each point represents the mean of determinations on the livers of four animals.

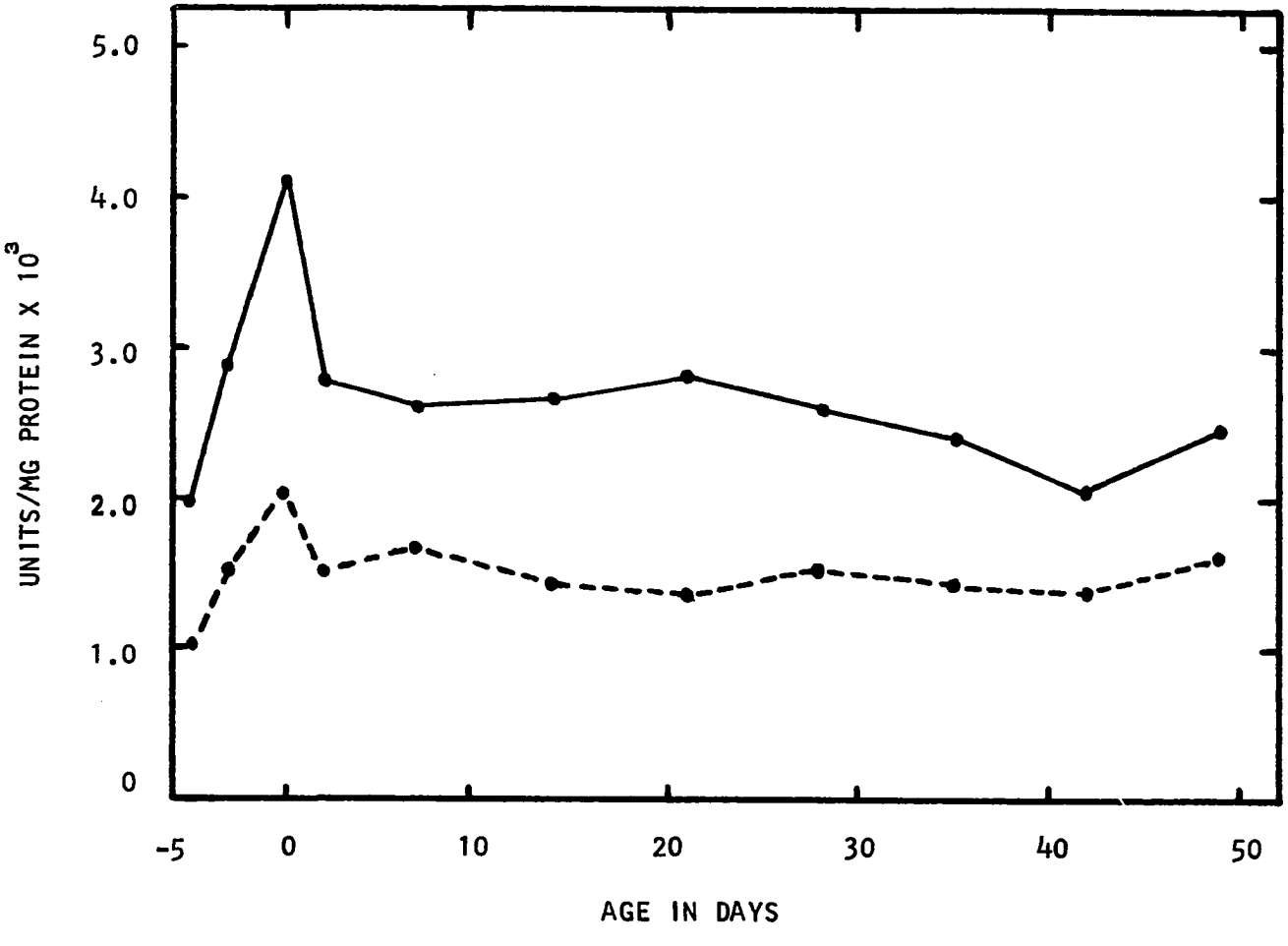
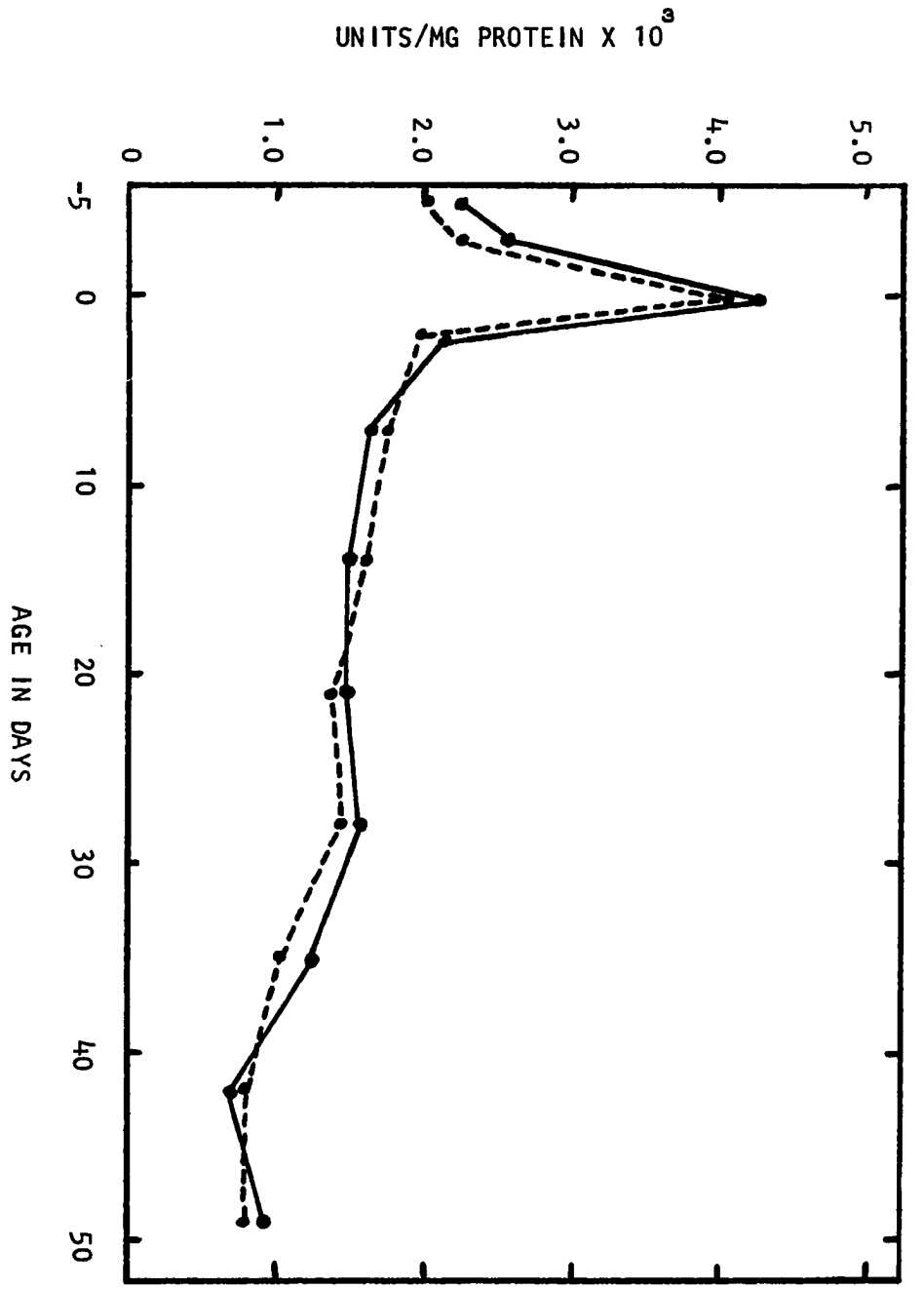

5.0
4.0
3.0
2.0
1.0
0
UNITS/MG PROTEIN X $10^3$
-5
0
10
20
30
40
50
AGE IN DAYS

Figure 6. Epimerase activity in the livers of male (———) and female (– – – –) chicks. A unit of activity is defined as the amount of epimerase needed to form 1 μmole of UDP glucose per minute at 30° C. Each point represents the mean of determinations on the livers of four aminals.


UNITS/MG PROTEIN X 10³
0
1.0
2.0
3.0
4.0
5.0
-5
0
10
20
30
40
50
AGE IN DAYS

galactokinase or for epimerase activities. However, transferase activity differed significantly ($P \leq 0.01$) between males and females throughout the study. Female chicks consistently showed 40 - 50% less transferase activity than males from five days before hatching through fifty days of age. It was concluded from this experiment that decreased toxicity with increasing age as judged by mortality was not due to an increase in the activity of the galactose metabolizing enzymes.

The next experiment was designed to determine the activities of the galactose metabolizing enzymes while the chicks were fed the 18% galactose diet. The activities of galactokinase, transferase and epimerase showed no significant differences from those of the control animals (Table 4). The author concluded that there was no induction of these enzymes after being challenged with the 18% galactose diet for 10 days at six weeks of age.

## Recovery and Comparison of Tissue Extraction Procedures

Recovery studies were done to establish the reliability of substrate quantitation for brain glucose, galactose and galactose-1-P and blood glucose and galactose. Exogenous substrate was added to the frozen brain tissue in the homogenizing buffer for brain determinations and to the whole serum for blood determinations. Appropriate controls were also conducted to correct for endogenous levels of brain and blood glucose. The results of these recovery studies may be found in Table 5. The recovery of exogenous glucose, galactose and galactose-1-P from brain homogenates ranged from 94.5% to 102.3%. The recovery of exogenous glucose and galactose from serum ranged from 94% to 98%. The

TABLE 4

ACTIVITIES OF THE GALACTOSE-METABOLIZING ENZYMES IN THE LIVERS OF SIX WEEK OLD CHICKS AFTER FEEDING THE 18% GALACTOSE DIET FOR TEN DAYS

| Enzyme | Male | | Female | |
|---|---|---|---|---|
| | Experimental | Control | Experimental | Control |
| Galactokinase (units x $10^3$/mg protein)[a] | 4.92 | 5.19 | 5.18 | 5.63 |
| Transferase (units x $10^3$/mg protein)[a] | 2.29 | 2.33 | 1.14 | 1.09 |
| Epimerase (units x $10^3$/mg protein)[a] | 0.92 | 0.84 | 0.80 | 0.85 |

[a]A unit is defined as the amount of enzyme needed to convert 1 μmole of substrate to product per minute at 30°C.

TABLE 5

RECOVERY STUDY FROM BRAIN TISSUE AND BLOOD FROM CHICKS FOR GLUCOSE, GALACTOSE AND GALACTOSE-1-P

| Tissue | Substrate | Amount of substrate added with homogenizing buffer or to Serum | Trials | % Recovery |
|---|---|---|---|---|
| Brain | Glucose | 1.7 μmole/g wet wt | 1 | 97.2 |
| Brain | Glucose | 3.3 μmole/g wet wt | 3 | 102.3 |
| Brain | Glucose | 8.3 μmole/g wet wt | 2 | 95.5 |
| Brain | Galactose | 4.0 μmole/g wet wt | 2 | 101.8 |
| Brain[a] | Galactose-1-P | 0.4 μmole/g wet wt | 2 | 94.5 |
| Blood | Glucose | 100 mg % | 2 | 97.5 |
| Blood | Galactose | 100 mg % | 2 | 96.8 |

[a]Mayes, unpublished results.

discrepancy from 100% may be due to endogenous synthesis or degradation of a substrate within the sample or to human error in the sample preparation.

A comparison of two extraction procedures was also performed. Ten three-day-old female chicks were fed the 18% galactose diet for one day. At this time the ten chicks were decapitated and the heads were dropped into liquid nitrogen. As each chick brain was disected from the skull, it was either placed into 2 volumes of cold 0.01M Tris-HCl buffer containing 0.14 M KCl at pH 7.8, homogenized, and heated for 5 minutes in a boiling water bath or the brain was placed into 2 volumes of cold 0.6 N perchloric acid (55) and homogenized. The homogenates were then centrifuged at 27,000 x g for 2 minutes at 0°C. The perchloric acid samples were then neutralized with 1.0 N potassium hydroxide solution containing 0.05 M potassium phosphate buffer at pH 7.5. These samples were then centrifuged to remove the insoluble potassium chlorate from the sample. The tissue supernates from both groups were then assayed for glucose, galactose and galactose-1-P as described in the Materials and Methods section (Chapter II). The comparison of the results of the two extraction methods may be seen in Table 6.

Glucose values were similar for both extraction methods. Galactose values were slightly different but variations between chicks is apparent from the large standard deviations. Galactose-1-P values from the Tris-KCl buffer were 78% greater than those samples from the perchloric acid extraction. This large difference may be due to differences between the chicks but it may also be due to sample preparation with concurrent degradation or synthesis of the substrate.

TABLE 6

COMPARISON OF TISSUE EXTRACTION PROCEDURE

| Substrate | $HClO_4$ extraction[a] µmole/g wet wt. | Tris-HCl, KCl pH 7.8[b] µmole/g wet wt. | P value[c] |
|---|---|---|---|
| Glucose | 1.91 ± 0.29 | 1.81 ± 0.13 | 0.80 |
| Galactose | 3.80 ± 1.48 | 4.51 ± 0.41 | 0.70 |
| Galactose-1-P | 0.151 ± 0.031 | 0.268 ± 0.018 | 0.05 |

[a]0.6 N $HClO_4$ neutralized with 1.0 N KOH containing 0.05 M $KPO_4$ buffer pH 7.5, Lowry (55).

[b]0.01 M Tris-HCl buffer containing 0.14 M KCl at pH 7.8.

[c]from t values for student's t test for non-paired varients for 5 animals from each tissue extraction buffer.

One can theoretically calculate the amount of galactose-1-P formed or degraded during the Tris-KCl tissue preparation. The following calculation is based on the reported amount of galactokinase in chick brain (33) for 6 minutes (time for homogenization and boiling). The calculated value is 0.027 μmole/6 min/g which is similar to the smallest value for galactose-1-P found in Tables 7 - 9 and is about 6% of the highest value for galactose-1-P.

Blood and Brain Glucose and Galactose and Brain Galactose-1-P

Information from the developmental enzyme study and the induction experiment did not account for the increasing tolerance to galactose with age. The next experiment was designed to assess changes in the absorption of glucose and galactose from the gut and also to determine if there were any changes in the accumulation of these two sugars and galactose-1-P in the brains of chicks fed galactose. As in the galactose toxicity study, the chicks were placed on the 18% galactose diet at day one, 21 and 42 days of age.

Blood glucose values (Tables 7 - 9) for each of the three phases were within the normal range for chicks. There were no significant differences between the glucose values for galactose-fed chicks and controls; nor were glucose values significantly different between males and females or for the three age groups studied (figure 7).

Blood galactose was observed only in galactose-fed chicks. It peaked on different days depending upon the age of the chicks being studied. For the day-old chicks the highest levels of blood galactose

TABLE 7

CONCENTRATIONS OF BLOOD GLUCOSE AND GALACTOSE AND BRAIN GLUCOSE, GALACTOSE GALACTOSE-1-P IN NEWLY HATCHED CHICKS

| SEX | AGE DAYS | DIET | DAYS ON DIET | NO. OF CHICKS | BRAIN | | | BLOOD | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | GLUCOSE µmole/g | GALACTOSE µmole/g | GAL-1-P µmole/g | GLUCOSE mg% | GALACTOSE mg% |
| M | 3 | C | 1 | 5 | 2.71±0.22 | - | - | 266±18 | - |
| F | 3 | C | 1 | 5 | 2.74±0.17 | - | - | 328±46 | - |
| M | 3 | E | 1 | 5 | 1.88±0.10 | 5.29±0.44 | 0.303±0.005 | 283±26 | 212±9 |
| F | 3 | E | 1 | 5 | 1.75±0.08 | 3.84±0.18 | 0.327±0.007 | 278±6 | 249±32 |
| M | 4 | C | 2 | 4 | 2.92±0.13 | - | - | 297±23 | - |
| F | 4 | C | 2 | 4 | 2.91±0.04 | - | - | 259±12 | - |
| M | 4 | E | 2 | 5 | 1.89±0.13 | 3.57±0.27 | 0.264±0.009 | 261±8 | 227±22 |
| F | 4 | E | 2 | 5 | 1.79±0.10 | 4.31±0.50 | 0.426±0.010 | 277±7 | 233±25 |
| M | 5 | C | 3 | 4 | 2.83±0.23 | - | - | 248±14 | - |
| F | 5 | C | 3 | 4 | 2.65±0.10 | - | - | 292±20 | - |
| M | 5 | E | 3 | 4 | 1.42±0.00 | 3.73±0.20 | 0.300±0.006 | 262±12 | 309±10 |
| F | 5 | E | 3 | 3 | 1.80±0.04 | 4.50±0.28 | 0.372±0.009 | 295±17 | 332±17 |
| M | 6 | C | 4 | 4 | 1.64±0.03 | - | - | 260±16 | - |
| F | 6 | C | 4 | 5 | 1.79±0.00 | - | - | 282±17 | - |
| M | 6 | E | 4 | 4 | 1.75±0.10 | 0.87±0.52 | 0.318±0.007 | 255±6 | 178±20 |

TABLE 8

CONCENTRATIONS OF BLOOD GLUCOSE AND GALACTOSE AND BRAIN GLUCOSE, GALACTOSE AND GALACTOSE-1-P IN THREE WEEK OLD CHICKS

| SEX | AGE | DIET | DAYS ON DIET | NO. OF CHICKS | BRAIN | | | BLOOD | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | GLUCOSE µmole/g | GALACTOSE µmole/g | GAL-1-P µmole/g | GLUCOSE mg% | GALACTOSE mg% |
| M | 22 | C | 1 | 5 | 2.91±0.24 | - | - | 298±7 | - |
| F | 22 | C | 1 | 5 | 3.21±0.18 | - | - | 279±5 | - |
| M | 22 | E | 1 | 5 | 1.94±0.17 | 2.31±0.59 | 0.144±0.004 | 307±14 | 219±47 |
| F | 22 | E | 1 | 5 | 2.28±0.09 | 4.17±0.80 | 0.405±0.009 | 312±9 | 255±39 |
| M | 23 | C | 2 | 5 | 2.15±0.26 | - | - | 317±21 | - |
| F | 23 | C | 2 | 5 | 2.12±0.08 | - | - | 300±5 | - |
| M | 23 | E | 2 | 5 | 1.73±0.07 | 3.17±0.28 | 0.276±0.009 | 283±9 | 265±14 |
| F | 23 | E | 2 | 5 | 1.92±0.11 | 4.40±0.43 | 0.387±0.007 | 304±9 | 294±68 |
| M | 24 | C | 3 | 5 | 2.00±0.12 | - | - | 299±15 | - |
| F | 24 | C | 3 | 5 | 2.17±0.12 | - | - | 289±14 | - |
| M | 24 | E | 3 | 5 | 1.61±0.09 | 1.39±0.26 | 0.240±0.005 | 273±10 | 172±18 |
| F | 24 | E | 3 | 5 | 1.41±0.17 | 2.52±0.47 | 0.378±0.006 | 284±5 | 199±21 |
| M | 25 | C | 4 | 5 | 1.97±0.22 | - | - | 297±14 | - |
| F | 25 | C | 4 | 5 | 1.73±0.09 | - | - | 317±17 | - |
| M | 25 | E | 4 | 5 | 1.25±0.07 | 0.97±0.08 | 0.240±0.003 | 252±6 | 152±22 |
| F | 25 | E | 4 | 5 | 0.93±0.12 | 1.41±0.33 | 0.378±0.007 | 276±11 | 68±5 |

TABLE 9

CONCENTRATIONS OF BLOOD GLUCOSE AND GALACTOSE AND BRAIN GLUCOSE, GALACTOSE AND GALACTOSE-1-P IN SIX WEEK OLD CHICKS

| SEX | AGE | DIET | DAYS ON DIET | NO. OF CHICKS | BRAIN | | | BLOOD | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | GLUCOSE µmole/g | GALACTOSE µmole/g | GAL-1-P µmole/g | GLUCOSE mg% | GALACTOSE mg% |
| M | 43 | C | 1 | 5 | 2.63±0.34 | - | - | 303±9 | - |
| F | 43 | C | 1 | 5 | 2.30±0.21 | - | - | 297±5 | - |
| M | 43 | E | 1 | 5 | 1.75±0.15 | 4.41±0.26 | 0.17±0.06 | 385±6 | 289±12 |
| F | 43 | E | 1 | 5 | 1.66±0.10 | 4.14±0.55 | 0.22±0.07 | 291±7 | 233±21 |
| M | 44 | C | 2 | 5 | 2.28±0.10 | - | - | 241±8 | - |
| F | 44 | C | 2 | 5 | 1.75±0.13 | - | - | 245±7 | - |
| M | 44 | E | 2 | 5 | 1.25±0.16 | 0.16±0.00 | 0.03±0.00 | 237±3 | 0±0 |
| F | 44 | E | 2 | 5 | 1.08±0.07 | 0.79±0.03 | 0.11±0.01 | 230±3 | 44±24 |
| M | 45 | C | 3 | 5 | 1.52±0.07 | - | - | 248±2 | - |
| F | 45 | C | 3 | 5 | 1.59±0.01 | - | - | 241±3 | - |
| M | 45 | E | 3 | 5 | 1.27±0.05 | 1.42±0.27 | 0.14±0.00 | 246±9 | 172±24 |
| F | 45 | E | 3 | 5 | 1.01±0.08 | 1.56±0.64 | 0.27±0.01 | 250±11 | 122±26 |
| M | 46 | C | 4 | 5 | 1.61±0.04 | - | - | 255±5 | - |
| F | 46 | C | 4 | 5 | 1.56±0.12 | - | - | 215±12 | - |
| M | 46 | E | 4 | 5 | 1.09±0.01 | 0.73±0.38 | 0.23±0.01 | 229±14 | 105±15 |
| F | 46 | E | 4 | 5 | 1.21±0.08 | 0.87±0.27 | 0.26±0.01 | 220±7 | 104±10 |

Figure 7. Blood glucose for control male chicks (0 - - 0), galactose fed males (0 —— 0), control female chicks (● - - ●), and galactose fed females (● —— ●) with increasing age.

Chicks were started on diet at 2, 21, or 42 days of age and continued for four days. Each point represents an average value for the four days on diet.

mg % BLOOD GLUCOSE
200
300
400
0
10
20
30
40
50
AGE IN DAYS

were reached after three days on the galactose diet. The highest levels of the 21-day-old chicks were on day two of feeding galactose and the 42-day-old chicks had the highest blood galactose levels on the first day of feeding galactose. These differences in the peak galactose concentrations are partially due to problems inherent in this type of experiment. Older chicks may initially consume more feed and become sick more quickly. This results in a decrease in appetite and thus a decrease in blood galactose. Blood glucose, however, is regulated and will fluctuate much less than blood galactose. There were significant decreases in blood galactose from the first to the fourth day of feeding galactose in the 21 and 42-day-old chick, but the blood galactose values for males vs females were not significantly different within each of the three phases. The values for blood galactose significantly dropped with age from the day-olds to the 42-day-old chicks ($P \leq 0.01$) (figure 8).

The values for brain glucose are given in Tables 7 - 9. For the three phases, concentrations of brain glucose in chicks on the control diet were significantly ($P \leq 0.01$) higher than chicks on the galactose diet. The brain glucose values also decreased significantly ($P \leq 0.01$) from the first phase of feeding to the third phase for both the controls and the experimental chicks (figure 9). There were no differences between males and females of the control groups or between males and females of the experimental group.

Galactose measured by the galactose dehydrogenase method could only be detected in the brains of experimental animals (Tables 7 - 9). There were no significant differences in the concentrations of brain

Figure 8. Blood galactose for galactose-fed female chicks (● - - ●) and galactose-fed male chicks (x—— x) with increasing age. Galactose feeding was started at 2, 21, or 42 days of age and continued for four days. Each point represents an average value for the four days on diet.

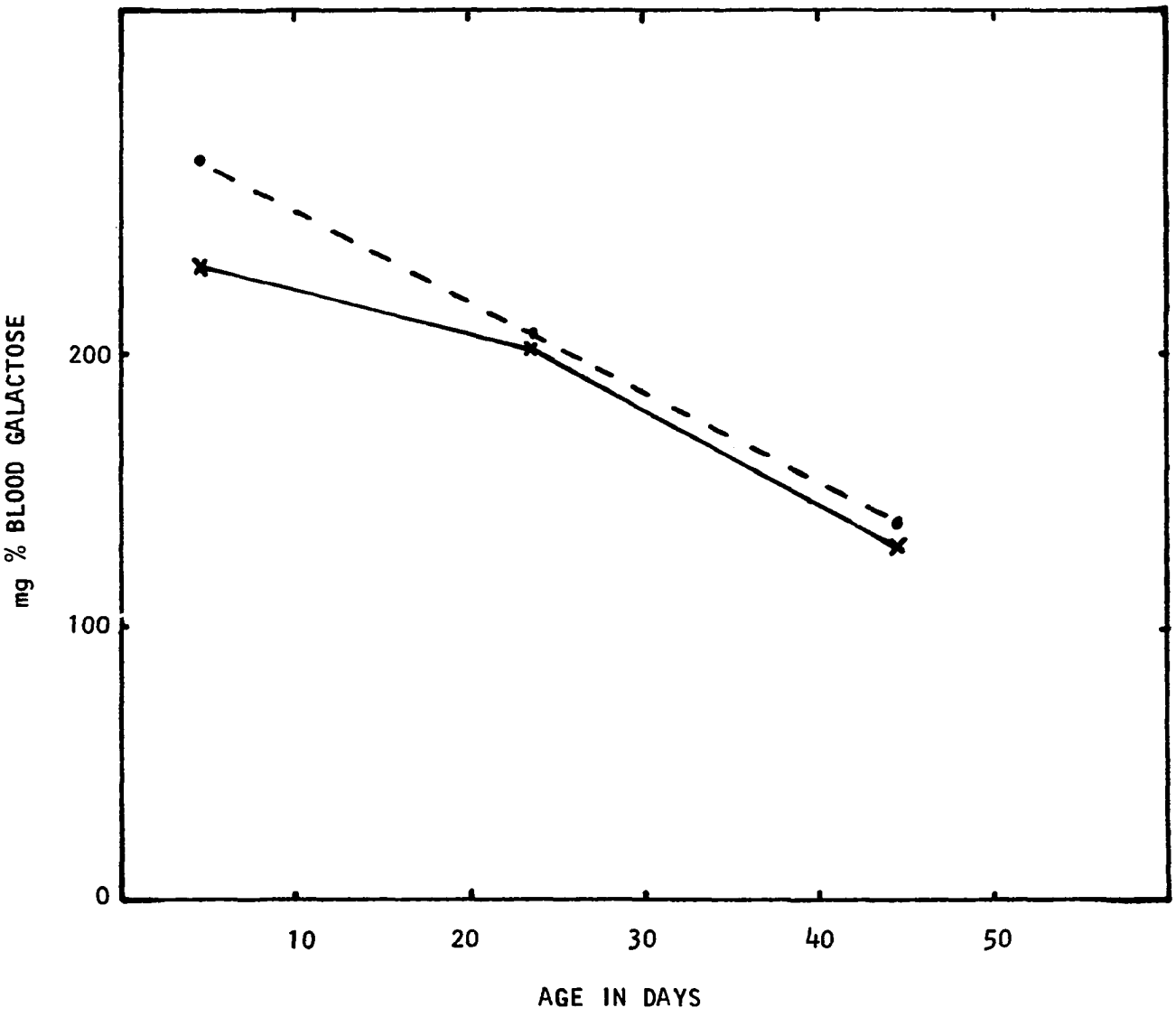
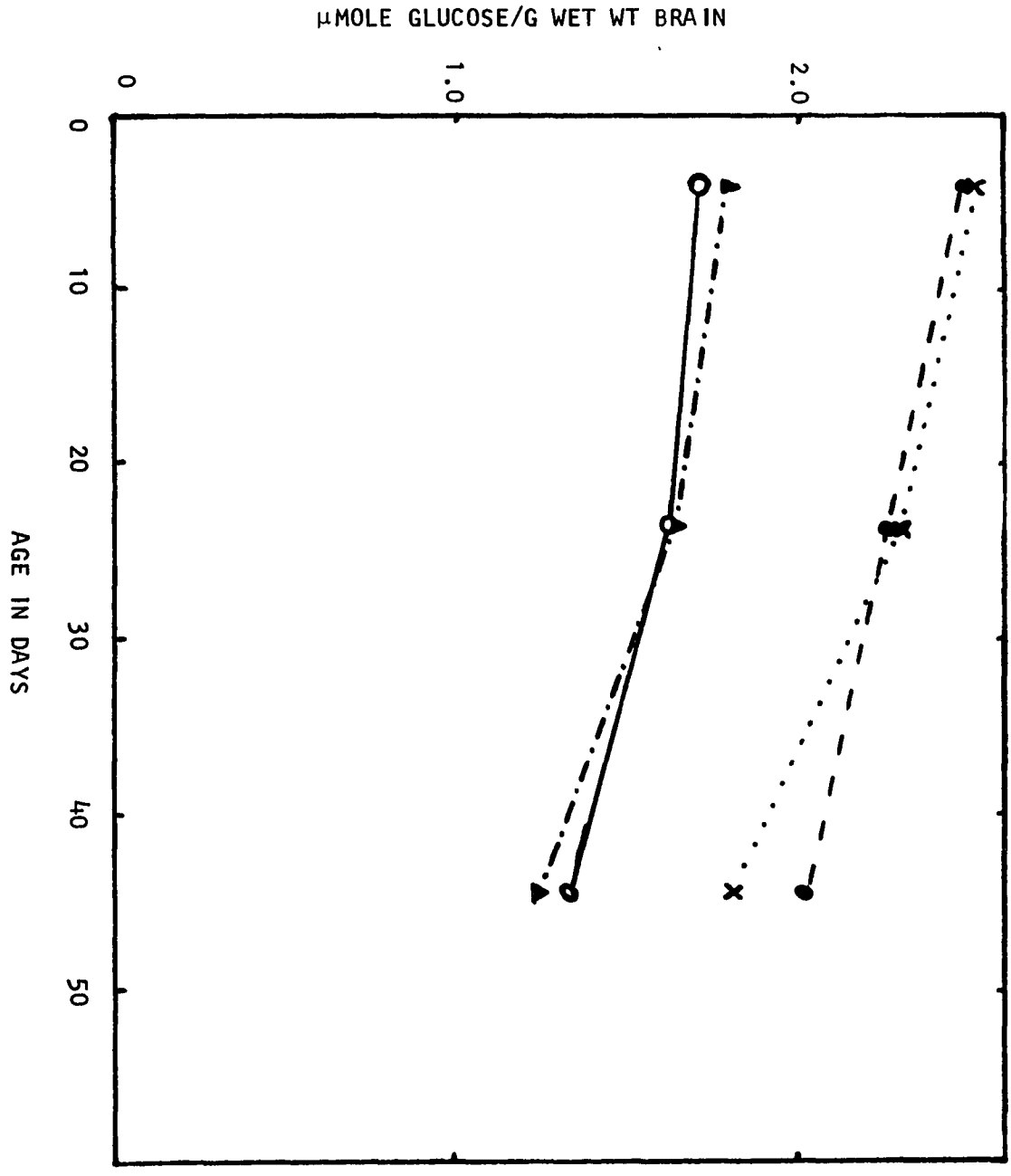

mg % BLOOD GALACTOSE
200
100
0
10
20
30
40
50
AGE IN DAYS

Figure 9. Brain glucose for control male chicks (● - - - ●), galactose-fed males (o —— o), control female chicks (X · · · X), and galactose-fed females (▲ - · - ▲) with increasing age.

Dietary regimen was started at 2, 21 and 42 days of age and continued for four days. Each point represents an average value for the four days on diet.


µMOLE GLUCOSE/G WET WT BRAIN
0
1.0
2.0
AGE IN DAYS
0
10
20
30
40
50

galactose between galactose-fed males and females; however, there were significant decreases ($P \leq 0.01$) in brain galactose in both male and female chicks from the first phase to the third phase of galactose feeding (figure 10).

Galactose-1-P concentrations in the brains of chicks were also measured in this study (Tables 7 - 9). There was no detectable galactose-1-P in the brains of control chicks. The values for galactose-1-P in brains of galactose-fed chicks were significantly different between males and females for the first two phases, day-olds and 21-day-olds ($P \leq 0.01$). However, with increasing age, significance between male and female galactose-1-P values for the 42-day-old chicks diminished. Brain galactose-1-P concentrations significantly decreased ($P \leq 0.01$) with the increasing age of the galactose-fed chicks from the first to the third phases (figure 11).

## Purification of Galactose-1-phosphate Uridyl Transferase

### Step 1. Crude Enzyme

Male and female chicks were fed commercial chick feed for six to eight weeks at which time they were sacrificed. The livers were removed, frozen in dry ice and stored in the deep freeze.

The livers from either male or female chicks were partially thawed and homogenized in 2 volumes (w/v) of 0.03 M KOH containing 0.005 M EDTA in a one gallon Waring blendor at high speed for a total of 90 seconds. All subsequent operations were carried out at 0 - 5°C. To this homogenate 2% protamine sulfate (0.2 volumes v/v) was added and the suspension was stirred for 30 minutes. The suspension was then

Figure 10. Brain galactose for galactose-fed females (▲ - - - ▲) and galactose-fed males (● —— ●) with increasing age. Chicks were started on galactose diet at 2, 21, or 42 days of age and continued for four days. Each point represents an average value for the four days on diet.

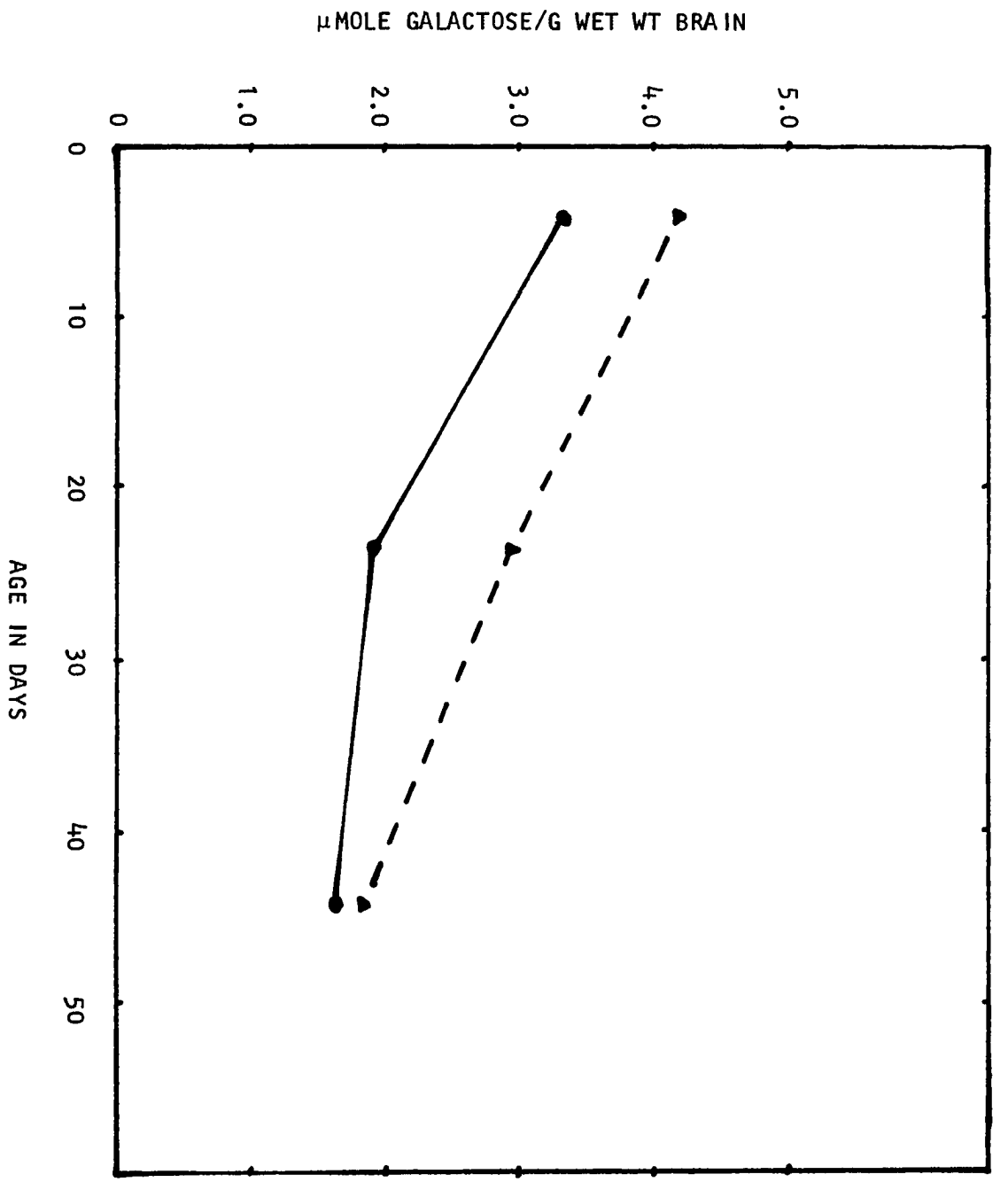
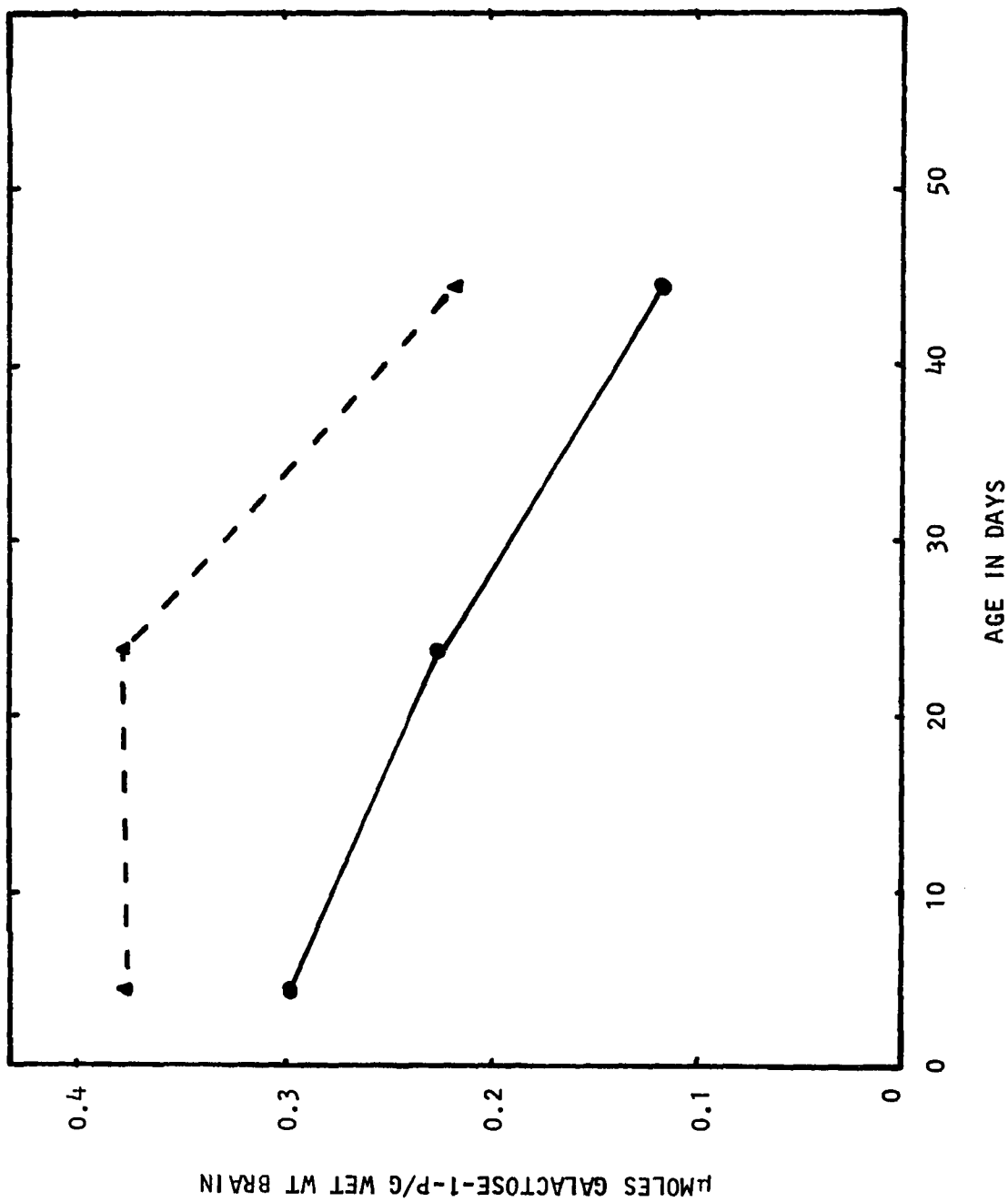

µMOLE GALACTOSE/G WET WT BRAIN
0
1.0
2.0
3.0
4.0
5.0
AGE IN DAYS
0
10
20
30
40
50

Figure 11. Brain galactose-1-P for galactose-fed females (▲ - - - ▲) and galactose-fed males (● —— ●) with increasing age. Chicks were started on galactose diet at 2, 21, or 42 days of age and continued for four days. Each point represents an average value for the four days on diet.


0.4
0.3
0.2
0.1
0
μMOLES GALACTOSE-1-P/G WET WT BRAIN
0
10
20
30
40
50
AGE IN DAYS

centrifuged at 27,000 x g for 30 minutes and the precipitate was discarded.

Step 2. Batch Treatment with Calcium Phosphate Gel

The supernate from step 1 was filtered through glass wool and 4 mg of calcium phosphate gel per mg protein was added. The resultant suspension was stirred for 40 minutes, centrifuged at 27,000 x g for 30 minutes and the supernatant was discarded. Four volumes (w/v determined from the initial amount of liver) of 0.04 M phosphate buffer pH 8.0 were then added to the calcium phosphate gel. This solution was homogenized two times for 15 seconds in a one gallon Waring blendor, stirred for one hour, and centrifuged at 27,000 x g for 30 minutes.

Step 3. DEAE Cellulose Chromatography

The supernatant from step 2 was diluted with distilled water containing enough 2-Mercaptoethanol (ME) and EDTA to result in a protein solution containing 0.025 M phosphate buffer pH 8.0, 0.01 M ME and 0.001 M EDTA. This solution was then applied to a DEAE cellulose (phosphate form) column (3 X 13 cm). The column was then washed with one liter of 0.05 M phosphate buffer pH 7.0 containing 0.01 M ME and 0.001 M EDTA until transferase activity began to be eluted at which time the buffer was changed in phosphate concentration to 0.07 M. All fractions containing over 0.015 units per ml were combined.

Step 4. DEAE Cellulose Chromatography

The combined effluent from step 3 was diluted with 1.5 volumes of 0.01 M ME and 0.001 M EDTA and allowed to run on a small (2 x 5 cm) DEAE cellulose (phosphate form) column. The activity was eluted with 0.16 M phosphate buffer pH 7.0 containing 0.01 M ME and 0.001 M EDTA.

All fractions containing 0.06 units per ml were combined.

Step 5. Hydroxylapatite Column Chromatography

The effluent from the above step was dialyzed against 60 volumes (v/v) of 0.001 M phosphate buffer pH 7.0 containing 0.01 M ME and 0.001 M EDTA three times for 1 hour each. The dialized enzyme solution was then applied to a hydroxylapatite column (1 ml bed volume/ mg protein). The column was washed with 0.001 M phosphate buffer pH 7.0 containing 0.01 M ME and 0.001 M EDTA. Transferase was eluted with 0.008 M phosphate buffer pH 6.8 containing 0.01 M ME and 0.001 M EDTA. Fractions containing more than 0.015 units per ml were combined.

Step 6. DEAE Cellulose Chromatography

The combined fraction from step 5 were applied to a small DEAE cellulose (phosphate form) column (1 X 3 cm) and eluted with 0.16 M phosphate buffer pH 7.0 containing 0.01 M ME and 0.001 M EDTA. Fractions containing 0.2 units/ml were combined and further concentrated using a Collodion bag apparatus (Schleicher and Schuell, Inc.). Male and female transferase enzymes were purified 1400 and 1100 fold respectively with a 25% yield. A summary of a typical purification experiment from 1 Kg of male chick liver can be found in Table 10.

## Molecular Weight Determination

The molecular weight of the purified transferases from both male and female were estimated by Sephadex G-200 gel filtration and by sucrose gradient centrifugation. Enzymes of known molecular weight were used in both methods for calibration. The sedimentation rate in a sucrose gradient of both transferases was compared to those of

TABLE 10

PURIFICATION PROCEDURE FOR GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE

FROM GALLUS DOMESTICUS LIVER

| Purification Step[a] | Volume ml | Total mg protein | Total enzyme units | Specific Activity (units/mg protein x $10^3$) | Fold Purification |
|---|---|---|---|---|---|
| 1. Crude enzyme after protamine sulfate | 2150 | 53,535 | 48.4 | 0.90 | |
| 2. Calcium Phosphate gel | 3200 | 10,000 | 46.81 | 4.68 | 5.2 |
| 3. First DEAE Cellulose | 790 | 284 | 45.03 | 158.00 | 175.5 |
| 4. Second DEAE Cellulose | 40 | 177 | 37.9 | 214.00 | 237.7 |
| 5. Hydroxylapatite | 360 | 22.5 | 22.1 | 982.00 | 1091.1 |
| 6. Third DEAE Cellulose | 14.5 | 12.73 | 12.88 | 1012.00 | 1124.6 |

[a]From 1000g of female Leghorn chick liver.

lactic dehydrogenase, horse liver and yeast alcohol dehydrogenase, hexokinase and peroxidase. Using the assumptions of Martin and Ames(53), the molecular weight of both transferases was estimated to be 88,500 $\pm$ 5000 (figure 12) based on the relative sedimentation rates for transferase and each of the standards. In the gel filtration experiments, the elution volumes from Sephadex G-200 were plotted against the log of the known molecular weights for the following enzymes: catalase, horse liver and yeast alcohol dehydrogenase, hexokinase and peroxidase. The elution volumes of both transferases were employed to estimate their molecular weights from this standard curve. The molecular weight for both transferases was approximately 85,000 $\pm$ 5000 (figure 13).

## Kinetics

The kinetic properties of the transferases purified from male and female chick liver were compared in an attempt to elucidate any differences between them. Representative Lineweaver-Burk plots of 1/v vs 1/S are given in figures 14 and 15. The Km and $V_{max}$ values for galactose-1-P and UDP glucose of transferase from male and female chick liver are given in Table 11. One may see from a comparison of these values that there appears to be little difference between the transferase from male and female chick liver. The Km values are similar for galactose-1-P and the values for $V_{max}$ for the same substrate appear to show a two fold difference between male and female. The Km values for UDP glucose also seem to show a two fold difference while the values for $V_{max}$ for the same substrate appeared to show a five fold difference. It would take further study with homogenous enzyme preparations to determine if

Figure 12. Sedimentation behavior in sucrose gradient centrifugation. Molecular weights are plotted versus distance migrated for yeast alcohol dehydrogenase (ADH-Y), lactic dehydrogenase (LDH), hexokinase (HEX), galactose-1-P uridyl transferase (TRANS), horse liver alcohol dehydrogenase (ADH-HL), and peroxidase (PER). This figure represents one of four experiments for purified transferase from male chick liver.

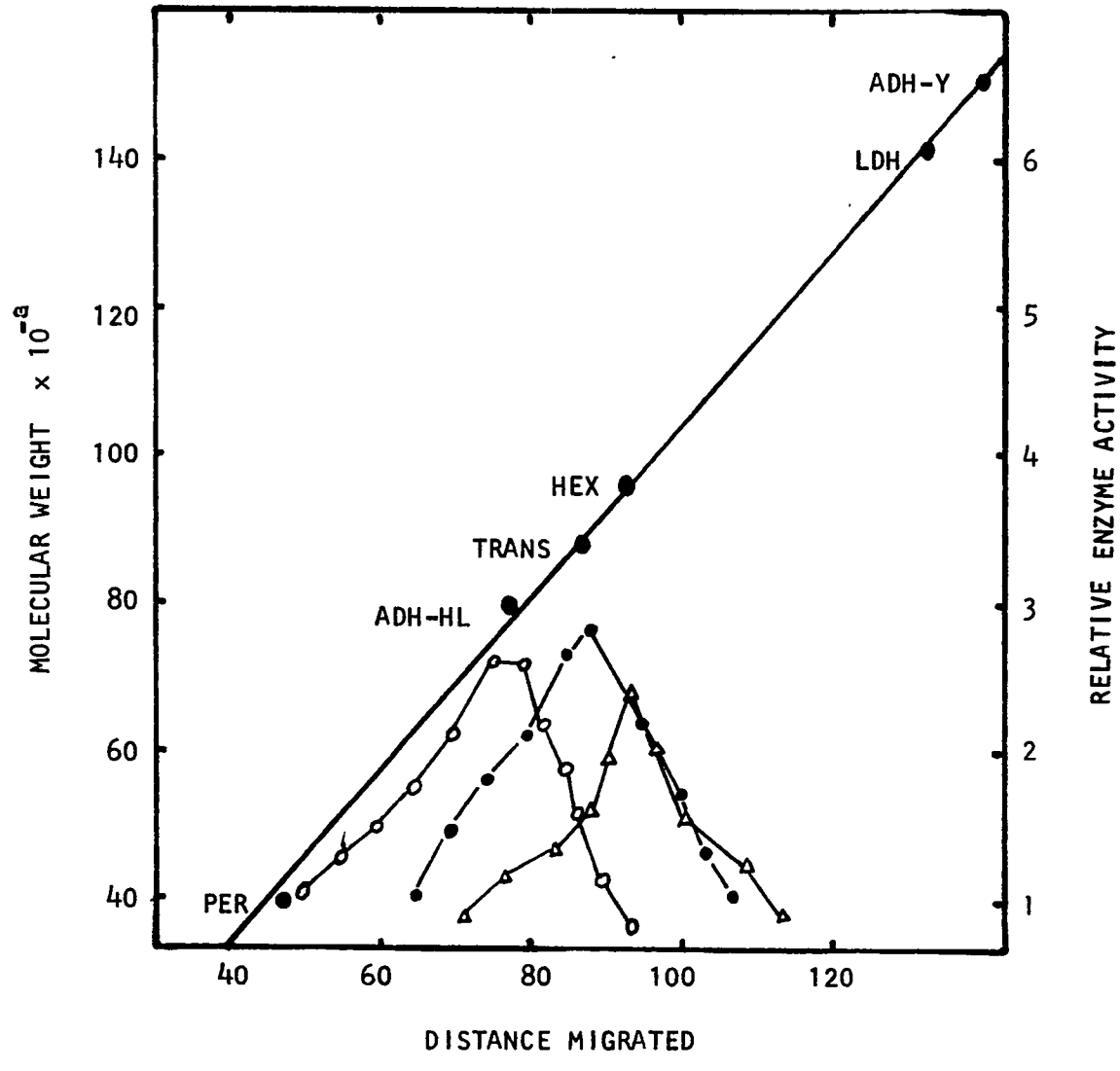

ADH-Y
LDH
HEX
TRANS
ADH-HL
PER
140
120
100
80
60
40
6
5
4
3
2
1
40
60
80
100
120
MOLECULAR WEIGHT x $10^{-a}$
RELATIVE ENZYME ACTIVITY
DISTANCE MIGRATED

Figure 13. Logarithmic plot by the method of Andrews (43) of Sephadex G-200 elution volumes versus the molecular weights of standards and the experimental molecule: catalase (CAT), yeast alcohol dehydrogenase (ADH-Y), lactic dehydrogenase (LDH), hexokinase (HEX), galactose-1-P uridyl transferase from female chick liver (TRANS), horse liver alcohol dehydrogenase (ADH-HL), and peroxidase (PER).

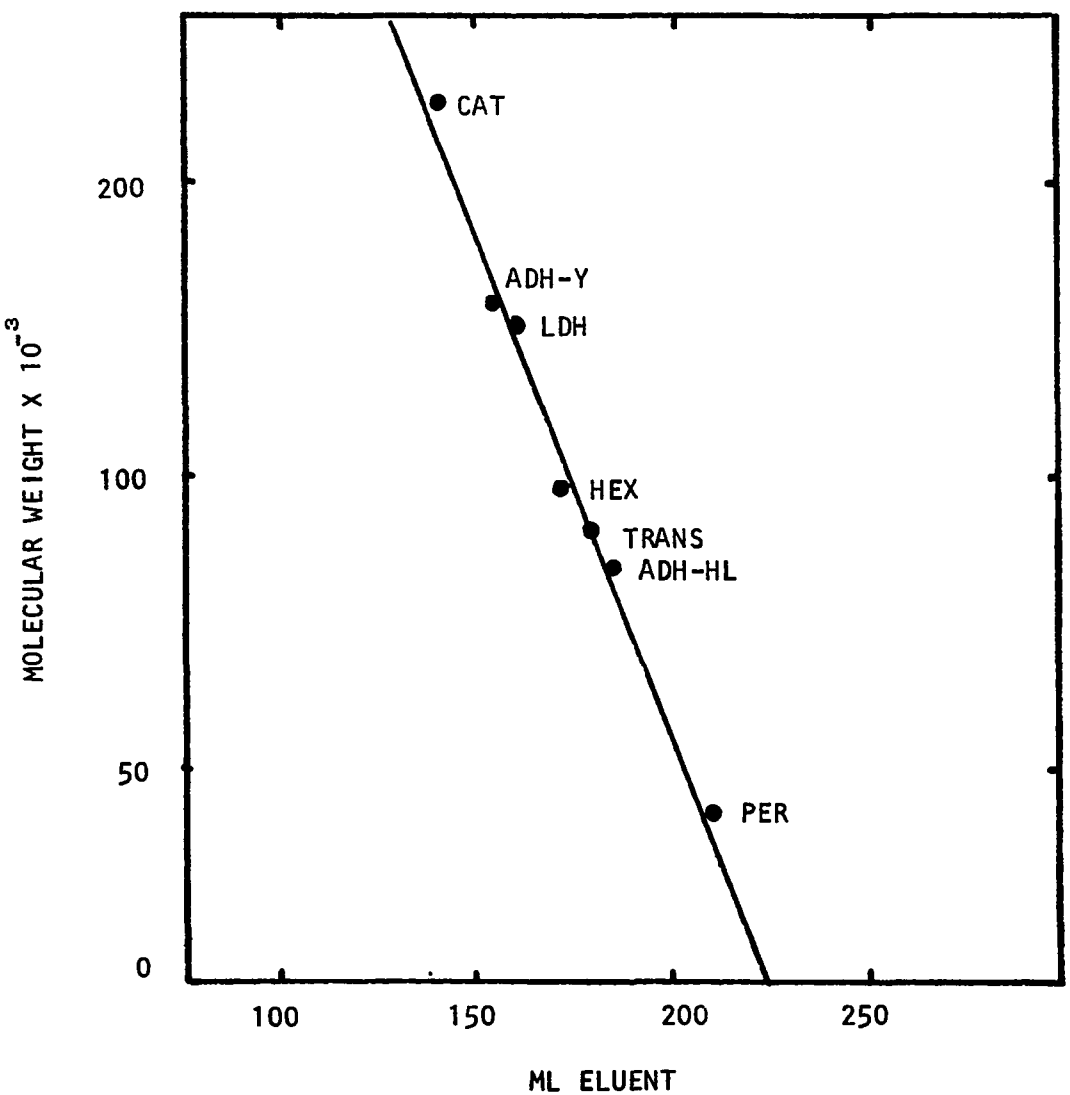

CAT
ADH-Y
LDH
HEX
TRANS
ADH-HL
PER
200
100
50
0
100
150
200
250
MOLECULAR WEIGHT X $10^{-3}$
ML ELUENT

Figure 14. A typical Lineweaver-Burk plot from one of four experiments of galactose-1-P uridyl transferase from male (- - -) and female (——) chicks with changing D-galactose-1-P concentrations.

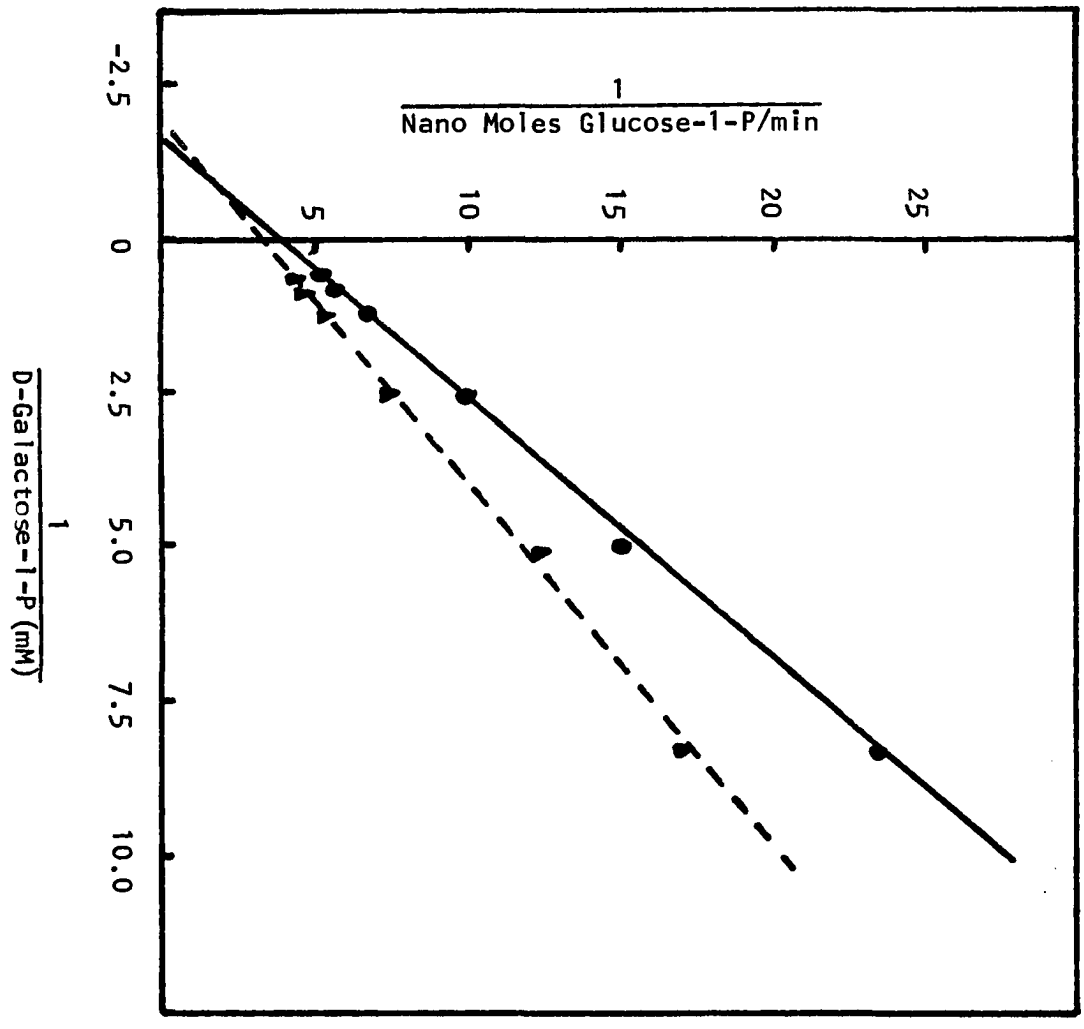

1
Nano Moles Glucose-1-P/min
5
10
15
20
25
-2.5
0
2.5
5.0
7.5
10.0
1
D-Galactose-1-P (mM)

Figure 15. A typical Lineweaver-Burk plot from one of four experiments of galactose-1-P uridyl transferase from male (- - -) and female (——) chick liver with changing UDP glucose concentrations.

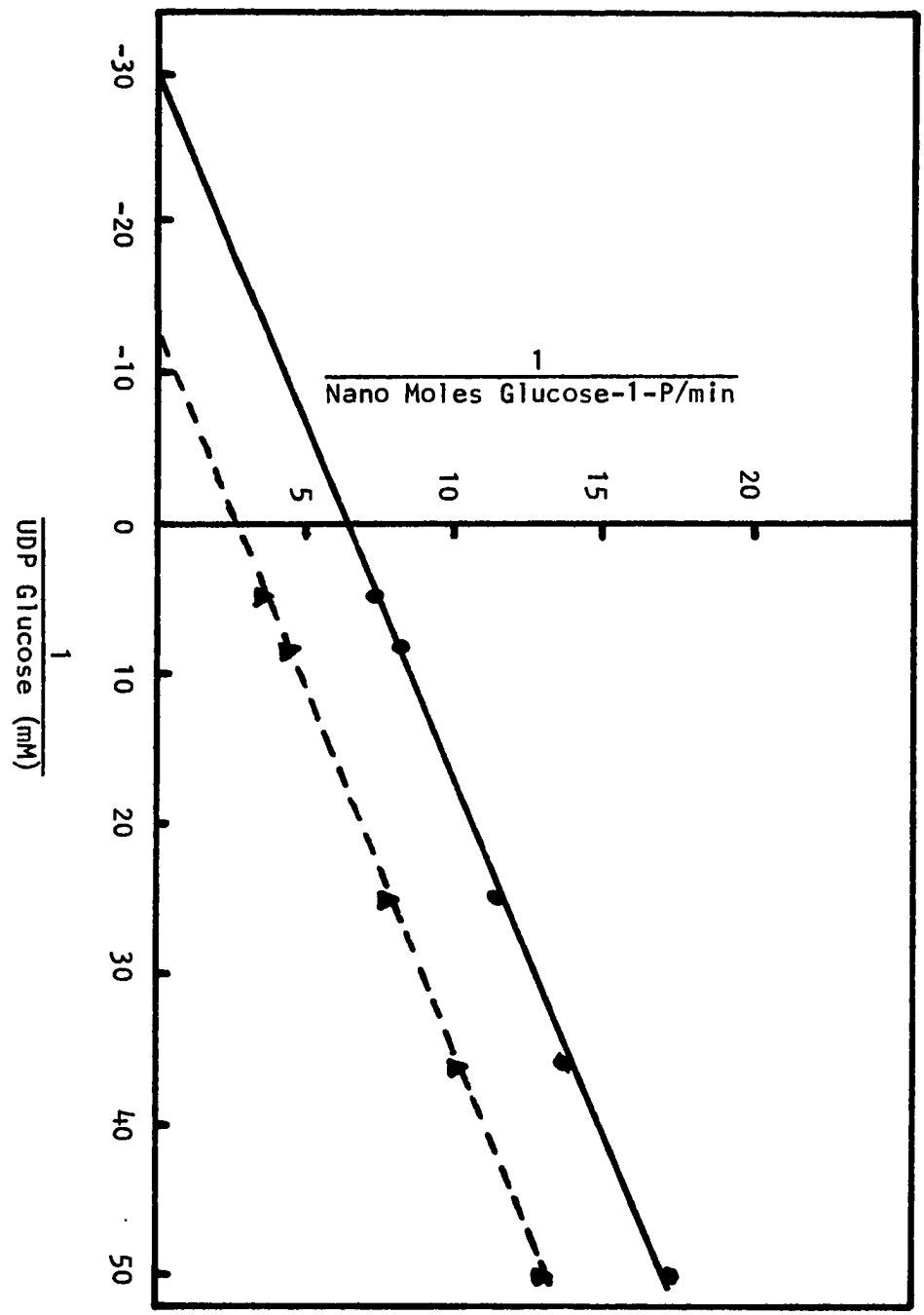

-30
-20
-10
0
10
20
30
40
50
1
UDP Glucose (mM)
1
Nano Moles Glucose-1-P/min
5
10
15
20

TABLE 11

COMPARISON OF VALUES FROM LINEWEAVER-BURK PLOTS FOR PARTIALLY PURIFIED TRANSFERASE FROM MALE AND FEMALE CHICK LIVER

| Sex | Specific Activity units/mg protein | Units ($\times 10^3$) used | Km (mM) | | $V_{max}$ (μmole/min/unit) | |
|---|---|---|---|---|---|---|
| | | | Gal-1-P | UDP Glc | Gal-1-P | UDP glc |
| F | 1.012 | 0.85 | 0.71 | 0.03 | 0.11 | 0.06 |
| M | 1.622 | 1.04 | 0.57 | 0.07 | 0.25 | 0.30 |

the differences found in these values for the two substrates between the transferases from male and female chicks are significantly different.

Temperature sensitivity profiles for the purified transferases are given in figure 16. Purified transferase from male and female chick liver demonstrated similar temperature sensitivities. They showed an increase in activity when incubated at 25° in 1% bovine serum albumin. This increase could possibly be due to a physical or chemical change conferred on the enzyme by the addition of protein or interaction with sulfhydryl groups in the bovine serum albumin molecule. Both enzymes were completely inactivated at 50°C for 5 minutes. The pH optima for both of the purified enzymes were also determined (figure 17). The two enzymes behaved almost identically with respect to changes in pH with optima between 8.5 and 8.9.

Figure 16. Temperature sensitivity of male galactose-1-P uridyl transferase from male ( —— ) and female ( - - - ) chick liver after prior incubation at various temperatures in 1% bovine serum albumin.

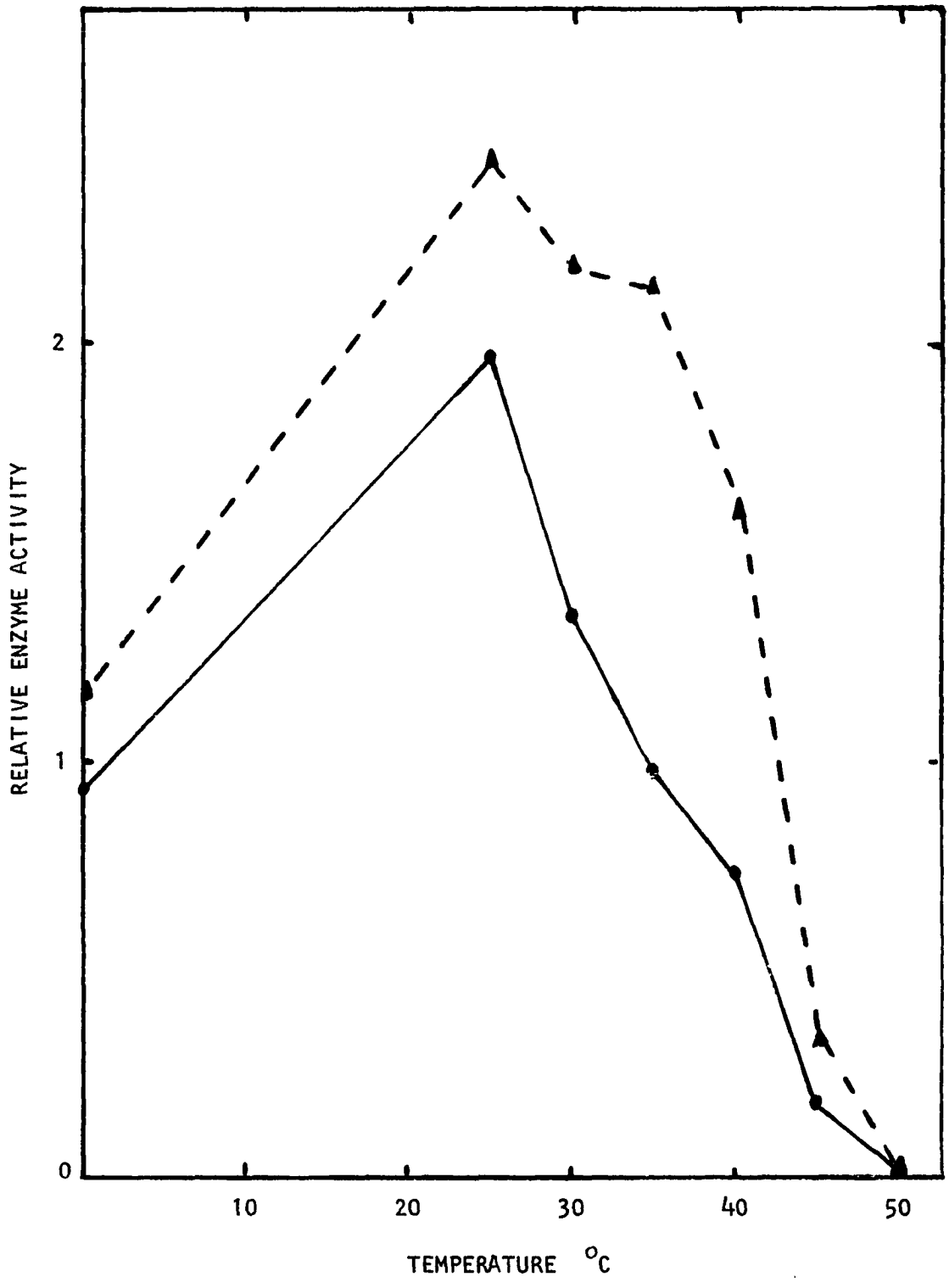
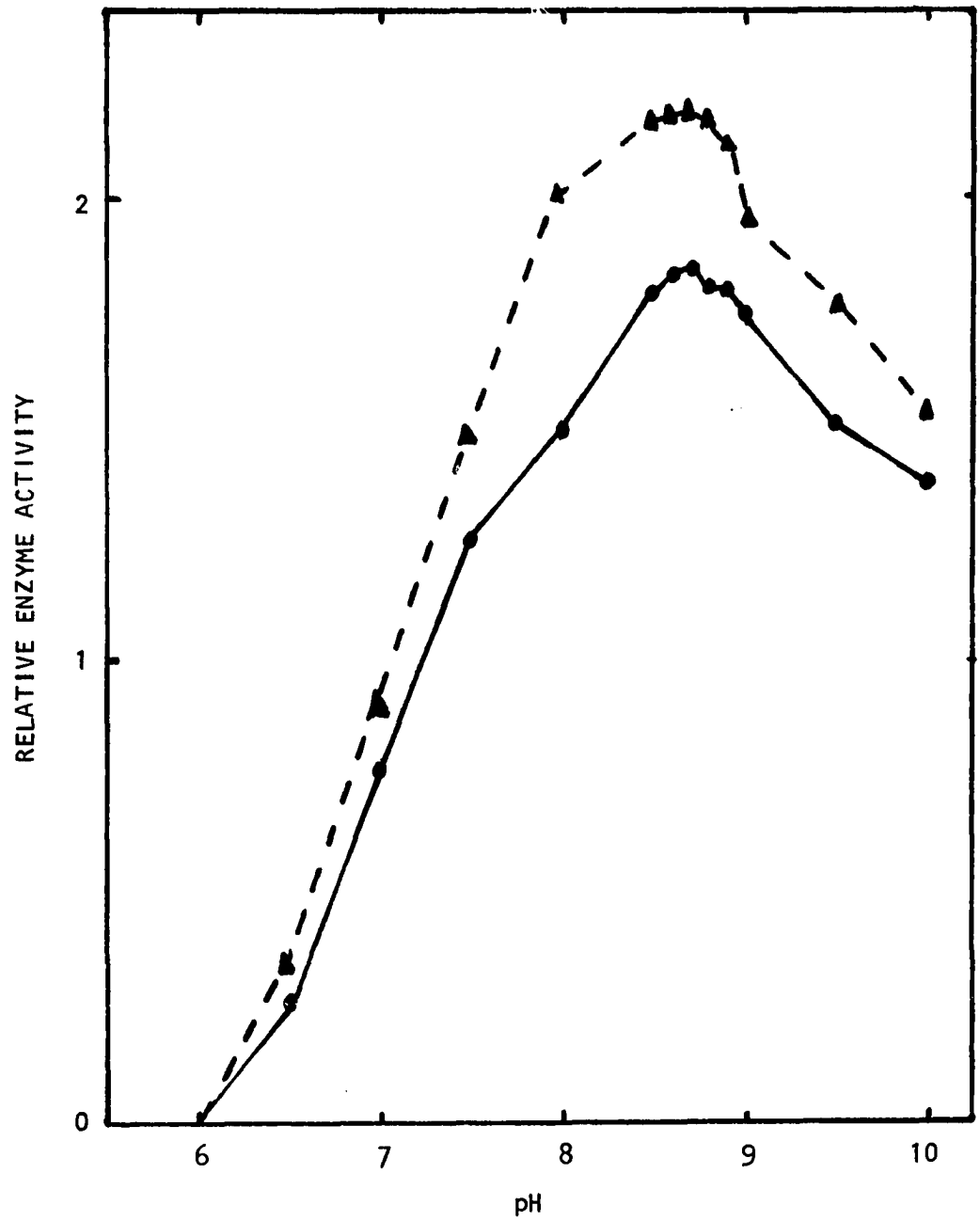

RELATIVE ENZYME ACTIVITY
2
1
0
10
20
30
40
50
TEMPERATURE °C

Figure 17. Enzyme activities of galactose-1-P uridyl transferase from male (——) and female (- - -) chick liver after incubation at several pH values.


RELATIVE ENZYME ACTIVITY
0
1
2
6
7
8
9
10
pH

# CHAPTER IV

## DISCUSSION

Older chicks more easily tolerate toxic amounts of dietary galactose than recently hatched chicks. This age-related tolerance has also been documented for galactosemic patients. It has been suggested that other enzymes capable of metabolizing galactose may develop with maturation. However, Donnell et al. (56) reported no evidence for increased galactose utilization with age, as judged by galactose load tests on the same galactosemic patients at different ages.

The increased tolerance for galactose with development of the chick found in this author's studies might be explained by accelerated galactose metabolism. Wells et al. (57) have shown that chicks metabolize galactose primarily be the Leloir pathway. Therefore, the development of these enzymes in the liver of chicks was studied. Maximal activity of galactokinase, transferase, and epimerase occurred at or near hatching. The activity rapidly declined and then remained relatively constant (40). These data contrast with data from developmental enzyme studies on rats, which showed liver galactose metabolizing to rise and peak shortly after birth and continue at relatively high levels until the time of weaning (58). However, since mammalian neonates normally ingest large amounts of galactose and chicks do not, such

developmental differences could be expected. After studying the oxidation of 1-$^{14}$C galactose in intact chicks of different ages, Wells et al. (57) also concluded that galactose metabolism appears to remain constant with advancing age in the chick. Since the ability to metabolize galactose appears to remain constant with advancing age in the chick, some other developmental process must be responsible for the increased tolerance to galactose with age.

This investigation indicated that there were no significant changes in the concentration of blood glucose between day-old chicks and those six weeks of age, but there was a trend for lower blood glucose in the six-week-old chicks than in the day-old or the three-week-old chicks. This is in agreement with Raheja (59) who demonstrated lower blood glucose in fourteen week-old chicks than in five week-old chicks. However, blood galactose decreases significantly ($P \leq 0.01$) during the same period. This could be caused by increased metabolism, decreased absorption, or increased excretion. Our studies and those of H.J. Wells et al. (57) indicate no increase in galactose metabolism with advancing age. Scharrer (60) in studies on trans-mucosal transport of glucose and galactose in the small intestine showed that chicks between two and seven weeks transported glucose at constant rates, but galactose transport decreased significantly during this period. Thus our studies and those of Scharrer suggest that chicks fed an 18% galactose diet would absorb less galactose with increasing age and demonstrated less mortality with maturation.

The brain concentration of glucose, galactose and galactose-1-P were all age dependent. These compounds decreased significantly ($P \leq 0.01$)

in the experimental animals from day-old chicks to chicks six-weeks of age. This decrease in the brain concentration of galactose and galactose-1-P might be expected because of a decrease in galactose transport across the small intestine and lower blood galactose values in the older chicks. But brain glucose also decreased significantly ($P \leq 0.01$) in the control and experimental chicks. This suggests that there may be a second phenomenon in addition to the development of an intestinal transport barrier to galactose. Studies using thiocyanate(61,62) radioactive chloride (61,62), glucose (63) and 5-hydroxytryptamine (64) indicate that there is development of a blood brain barrier in chicks with maturation and Lajtha (62)has reported that measurable development of this barrier occurs at about three weeks of age in chicks.

Galactose is much less toxic to male chicks than to female chicks (27,28,29). Previous studies have indicated that female chicks have 40-50% less liver transferase activity than males (33). Our experiments demonstrated that the liver transferase activity difference in chicks between males and females extends throughout development, from 5 days before hatching through 50 days of age. There was also no change in the activity of the transferase or of the epimerase and galactokinase after being fed for 10 days on the 18% galactose diet. Wells et al. (57) observed no disparity between male and female chicks in the conversion of $1^{14}C$ galactose to $^{14}CO_2$, but a difference might have emerged had larger quantities of galactose been used.

In another series of experiments the author attempted to elucidate the reason for the 40-50% difference in transferase activity between male and female chicks. Galactose-1-P uridyl transferase was purified from six week old male and female chick liver, 1400 and 1100 fold respectively. The molecular weights of the purified enzymes from both male and female chicks were 88,500 $\pm$ 5000 from sucrose gradient centrifugation and 85,000 $\pm$ 5000 from Sephadex gel filtration. These chick liver transferase molecular weights are about the same as those published for human liver, 90,000 (65) and Escherichia coli, 80,000 (66). The pH optima for the purified enzymes from both sexes were between 8.5 and 8.9. The temperature sensitivity results were very similar for both enzymes. Kinetic properties of both transferases were also studied. The Km values for galactose-1-P for partially purified transferase from male and female chicks were 0.57 mM and 0.71 mM respectively. The Km values for UDP glucose with transferase from male and female chicks appear to be different, 0.07 mM and 0.03 mM. $V_{max}$ values for UDP glucose were 0.03 and 0.62 µmole/min/unit for transferase from male and female. Though there appear to be differences between the Km values for UDP glucose and the $V_{max}$ values for galactose-1-P and UDP glucose, these would have to be studied more exhaustively on homogenous enzyme preparations to determine whether or not the differences are statistically significant. In summary the properties of male and female transferase appeared to be similar. The difference seen in transferase activities between the sexes may be due to a greater amount of enzyme in the male chick liver.

The concentrations of blood glucose and galactose and brain glucose, galactose and galactose-1-P were compared between male and female chicks. There were no significant differences between males and females in the blood glucose concentrations for any age studied. Female chicks demonstrated higher amounts of brain galactose than males but the difference between them decreased with age. Female chicks also showed higher amounts of brain galactose-1-P than males but this difference also decreased with age. Brain glucose concentrations from male controls and females were nearly identical as were those from galactose-fed males and females.

A number of parameters might result in and account for the neurotoxicity observed in galactose-fed chicks. Kozak and Wells (35) found after feeding a 40% galactose diet to newly hatched chicks that ATP levels were significantly lowered in the galactose-fed animals with a concomitant increase in ADP and AMP levels. After studying the turnover of galactose intermediates, they (32, 67) postulated the existence of a futile cycle, phosphorylation and dephosphorylation of galactose, which would account for the depletion of energy reserves. Knull and Wells (68) also found a temporary return to normal levels of ATP, phosphocreatinine, glucose, fructose-1-6 diphosphate and lactate, and recovery from the neurotoxicity after an intraperitoneal injection of 1.0 M glucose into the neurotoxic chicks. They also found that brain galactose, galactitol, and galactose-1-phosphate remained about the same after the injection of glucose. They therefore suggest that galactose interferes with glucose entry into the brain and this causes a decrease in the brain energy reserves. They imply that this may be the major cause of neurotoxicity in the chick (39). However,

Malone et al. (69) found no significant difference in ATP levels between brain from galactose-fed chicks and controls and they (37) suggest that serum hyperosmolality is the major cause of galactose toxicity in the chick. They (69) also demonstrated a decreased uptake of glucose by brain of galactose toxic chicks. Patients with galactokinase deficiency have elevated blood galactose, which would probably inhibit the transport of glucose across the blood-brain barrier as in the chick, but these individuals show no impaired mental function.

One of the purposes of this project was to define the molecular mechanism of neurotoxicity in the chick. The author's studies showed a significant decrease in the concentration of brain glucose with age for control and galactose-fed chicks. The difference between control and experimental chicks remained the same in all three age groups, but the mortality disappeared in the six week old chicks. Therefore, the lower glucose levels in brains of galactose-fed chicks may not be directly related to galactose neurotoxicity. There was a decrease in the brain galactose and galactose-1-P values from day-old through six week old chicks and there was a difference between male and female chicks. This difference decreased in the six-week-old chicks. Mortality followed the same pattern. These results on blood and brain concentrations of glucose, galactose, and galactose-1-P in relation to age and sex in the chick seem to indicate that there is a critical relationship between the concentrations of brain galactose and galactose-1-P and neurotoxicity. We favor the point of view that galactose-1-P is the neurotoxic agent.

# CHAPTER V

## SUMMARY

Galactosemia is the result of an inborn error in the metabolism of galactose in man. The following research was designed to characterize manifestations of galactose toxicity in an animal model.

When an 18% galactose diet was fed to Leghorn chicks, marked mortality differences occurred depending on sex and age. Galactose was found to be most toxic to newly hatched chicks, was less toxic to three-week-old chicks and was least toxic to six-week-old chicks, when each group was kept on the experimental diet for 10 days. In the first two groups females were consistantly more susceptible to galactose toxicity than males. Brain and blood glucose values were determined in control and experimental animals for each of the three age groups. Blood galactose and brain galactose and galactose-1-P concentrations were measured in experimental chicks for each of the three age groups. Concentrations of these compounds decreased in the brain and blood as the age of the chicks increased. Females showed higher brain galactose and galactose-1-P values than did males of the same age. Galactose-fed chicks had lower brain glucose values than controls of the same age.

Since these results suggest a rise in the galactose metabolism might accompany increasing age, the galactose metabolizing enzymes were determined in the livers of developing chicks. Galactokinase, galactose-1-P uridyl transferase, and UDP galactose-4-epimerase showed peak activities on or about the day of hatching for both males and females. These activities subsequently declined and then remained relatively constant. No consistent differences were apparent between male and female chicks in the activities of the liver galactokinase and epimerase. Transferase activity, on the other hand, was significantly higher in the liver of male chicks throughout development, from five days before hatching through fifty days of age.

Since developmental studies showed a two-fold higher transferase activity in liver from male chicks, the enzymes from male and female chicks were purified and characterized. The Michaelis constants of purified transferase enzymes for galactose-1-P and UDP glucose were nearly identical for both sexes. Molecular weights as judged by Sephadex gel filtration and sucrose gradients of both of the enzymes ranged from 85,000 to 88,500. There were no significant differences in temperature sensitivity and pH optima between transferases from male and female chick liver.

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