

EFFECTS OF GROWTH HORMONE, INSULIN, AND  
THYROXINE ON HEPATIC UDPG-GLYCOGEN GLUCOSYLTRANSFERASE,  
HEXOKINASE, AND GLUCOKINASE IN THE FETAL RAT

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## SUMMARY

Pregnant Sprague-Dawley-Holtzman rats were laparotomized on either the nineteenth or the twentieth days of gestation (ether anesthesia). The fetuses were injected in utero with growth hormone, insulin, or thyroxine. Saline-injected fetuses served as controls. Following the injections, the maternal abdominal wall was sutured and the skin closed with wound clips. Twenty-four hours later, the fetuses were removed and weighed. The livers were weighed and then assayed for glycogen. Thyroxine administration was found to have increased hepatic glycogen deposition.

These results prompted a study of the effect of hormone treatment on two enzymes active in the fetus which may serve as control points in fetal metabolism. These enzymes were UDPG-glycogen glucosyltransferase and hexokinase. Glucokinase was assayed concurrently with hexokinase.

Surgery similar to that described above was performed on pregnant Sprague-Dawley-Holtzman rats with the following changes. The pregnant females were laparotomized and the fetuses injected on either the fifteenth or the nineteenth days of gestation. Fetuses received treatment identical to fetuses in the glycogen experiment. However, each liver was assayed for independent(I) and dependent (D) UDPG-glycogen glucosyl

transferase or for hexokinase and glucokinase.

The results of this experiment demonstrated that the effect of thyroxine administration on glycogen deposition late in gestation is not mediated through an increase in the activity of either the I or the D form of UDPG-glycogen glucosyltransferase. Instead, the effect may be achieved through increased availability of glucose for incorporation into glycogen or through alteration of cellular metabolites which may stimulate glycogenesis.

Glucokinase activity was found to be absent on the sixteenth and the twentieth days of gestation in controls as well as in hormonally treated livers.

Hexokinase activity was increased significantly at day 16 and day 20 by the injection of growth hormone. This increase may be mediated through a protein anabolic effect by this hormone. Insulin appeared to increase the activity of hexokinase at day 20, but an analysis of variance did not show a significant difference between the mean of the treatment activities and that of the control activities. A ranking test did show a significant difference between the activity means within mothers, indicating that there was indeed a bias. The treatment of more animals may show a significant effect by insulin on hexokinase activity.

## CHAPTER I

### INTRODUCTION

The purpose of this research was to demonstrate the effects of insulin, growth hormone, and thyroxine on hepatic uridine diphosphoglucose (UDPG)-glycogen glucosyltransferase, hexokinase, and glucokinase in the fetus. UDPG-glycogen glucosyltransferase plays a key role in the deposition of glycogen in the liver late in development. At birth the glycogen will be used to maintain a sufficient blood glucose level until the newborn's metabolism achieves the transition between prenatal glucose metabolism and postnatal lipid metabolism (1). The fetus derives its serum glucose primarily from the mother by means of the placenta and not via endogenous production. This glucose probably serves as a prime energy source for metabolism and as the main source for glycogenesis. The high activity of hexokinase before birth suggests a high capacity for phosphorylating glucose (2). Although liver glucokinase reputedly does not appear until weaning, it was assayed concurrently for the possible effects of prenatal hormone administration.

It has been found that key enzyme changes in mammalian liver are associated with specific developmental changes (3). Of particular importance are those changes occurring in the late fetal period (the sixteenth to the twentieth day of gestation) of development which prepare the fetus for the metabolic

changes at birth. The late fetal changes seen in the enzyme profiles of the liver occur concurrently with or just after the onset of pituitary, thyroid, adrenal cortical, and pancreatic  $\beta$ -cell function. The concurrence of the two events suggests that the rise in fetal hormone production may serve as a trigger for enzymic differentiation. Fetal injection of representative hormones appearing late in gestation may provide some insight into enzymic differentiation. Logical choices of hormones for fetal administration would be insulin, growth hormone, and thyroxine.

An increase in the activity of a certain enzyme following the administration of a certain hormone late in gestation (day 19) could give an indication of the function of the hormone in the fetus. Early enzymic differentiation resulting from hormone administration prior to the normal appearance of an enzyme (day 15) could indicate the hormone as a trigger, an important link in the initial control of development of a particular enzyme.

## CHAPTER II

### REVIEW OF THE LITERATURE

Enzymic differentiation has become the topic of intensive research in recent years. The term enzymic differentiation is used to indicate the process whereby organs acquire their characteristic enzyme patterns (4). Much interest has been shown in this process in liver tissue because of the importance of its enzymes in survival following birth. A survey of the data available on rat liver indicates discontinuous changes in the enzyme profile. Specific developmental stages are associated with extensive enzyme changes. Because enzyme changes tend to cluster in time, the specific enzymes are grouped according to the developmental stage in which they first appear.

The three stages of development which are associated with intense changes in enzyme activity are (a) the late fetal period, the sixteenth to the twentieth day of gestation; (b) the early neonatal period, the first day of birth; and (c) the late suckling period, the third week of postnatal life (4). These changes in enzymic differentiation occur along with changes in the hormone profile; the late fetal period of development is associated with the initiation of endocrine secretion of several glands. Studies with labeled

iodine uptake in the fetal rat thyroid indicate that the fetus is secreting thyroid hormone by the seventeenth day of gestation (5). Experiments by Jost have demonstrated evidence of thyroxine secretion before measurable iodine uptake, thereby suggesting that thyroid function may actually begin slightly before the seventeenth day (6). The adrenal gland develops in the rat between the twelfth and sixteenth day of gestation and is capable of corticosterone secretion on the fourteenth day (7). Adrenal secretion comes under trophic control on the sixteenth day of gestation with the beginning of pituitary function. Pituitary secretion does not seem to be necessary for early development of either the thyroid or the adrenal gland but does exert trophic control over the secretions of these glands late in gestation (1). The  $\beta$ -cells of the pancreas are functional on the twelfth day as demonstrated by the onset of insulin secretion at that time (8).

Jost was one of the first to experiment with the effects of fetal endocrine function on biochemical differentiation. His work has shown that hypophysectomy by decapitation of the fetal rabbit does influence biochemical differentiation (9). The liver does not produce and accumulate appreciable amounts of glycogen unless glucocorticoids and a pituitary extract are administered. Jacquot and Kretchmer later demonstrated that the enzymes required for fetal rat liver glycogen formation (especially UDPG-glycogen glucosyltransferase) fail to reach their customary high activities

after hypophysectomy unless glucocorticoids are administered (10). Such endocrine ablation studies have been helpful in indicating possible roles of endocrine products on differentiation; however, evaluation of the results of such experiments is limited. Endocrine ablation, especially when performed by fetal decapitation, may deprive the fetus of a host of hormones or substrates rather than of a single one. The technique of hormone administration to an intact fetus has recently become the method of choice. While endocrine ablation experiments can only indicate which factors are necessary for enzymic differentiation, hormone administration experiments may indicate the order of importance of those factors and which factor serves as the trigger for differentiation. On the whole, hormone administration in utero seems to be a more positive approach while inflicting minimum injury and trauma to the fetus.

In 1961 Jost postulated that the late phase of rapid glycogen accumulation in fetal liver depends on adrenal and pituitary hormones (11). In 1964, Grillo et al. demonstrated that initial glycogen deposition corresponds with the earliest detection of UDPG-glycogen glucosyltransferase and with the onset of insulin secretion on the twelfth day of gestation (12). Monder and Coufalik returned to the earlier experiments and found that cortisol stimulated the synthesis of glycogen in fetal rat liver explants (13); the applicability of this experiment to the in vivo situation may be questionable

because substrate availability and intracellular environment in an in vitro incubation may vary substantially from that found in vivo.

Greengard and Dewey found that administration of glucocorticoid to the intact fetus prior to the normal increase in glycogen accumulation induced early deposition of glycogen and thus may serve as a trigger (14). Recent experiments by Parsa have shown a positive correlation between levels of glucosyltransferase and the development of rough and smooth endoplasmic reticulum in chick liver (15). The absence of insulin in explants prevents the development of endoplasmic reticulum, the induction of glucosyltransferase, and the deposition of glycogen.

Less is known about the differentiation of hexokinase. Burch et al. found that hexokinase activity decreases from the fifteenth day of gestation until the twenty-first day after birth (16) (See Appendix B). Schaub et al. substantiated this conclusion (3). No evidence of the effects of prenatal hormone administration on hexokinase was found in the survey of the literature.

Glucokinase differentiation has been the object of much research. Walker reported that fetal guinea-pig liver contains a glucokinase which has a low  $K_m$  (17). No such enzyme has been reported in the fetal rat. Rat liver glucokinase does not appear until the time of weaning (sixteen to twenty days following birth) (18-22). The normal appearance of

glucokinase is triggered by insulin and glucose (23). Jambadar and Greengard have prematurely induced the appearance of glucokinase on the ninth day following birth by repeated injections of glucose and hydrocortisone (21).

Greengard and Dewey have performed several experiments involving the effects of prenatal hormone administration on other enzymes of the late fetal and early neonatal clusters. They have found that thyroxine elevates considerably the levels of glucose-6-phosphatase and NADPH, cytochrome c oxidoreductase when administered between the eighteenth and twenty-second days of gestation (24). When administered in the same way, glucagon has a similar effect on glucose-6-phosphatase and can prematurely evoke the synthesis of tyrosine aminotransferase and serine dehydratase, enzymes of the neonatal cluster (25). These results have led Greengard to postulate that the accumulation of new enzymes and gains or losses in the concentrations of enzymes already present late in gestation may be associated with the onset of thyroid function. Similarly, the enzymatic changes seen at birth may be associated with the onset of glucagon secretion as a result of neonatal hypoglycemia (4). While her theories are plausible, the experimental data might be questioned on the basis of the dosages of thyroxine administered (3  $\mu$ g. per fetus). The thyroxine dosage used with her fetuses has been shown to produce euthyroidism in thyroidectomized adult rats weighing 200 to 300 grams (26-28). Although a physiological

dose of thyroxine in a fetus has not been defined, one must be cautious when evaluating results of administration of what may be a pharmacological dose, a dose resulting in the levels of thyroxine being substantially higher than the normal endogenous levels.

Greengard's theories of the mechanisms of enzyme development are based on the development of competence (a state of responsiveness) of liver cells for enzyme synthesis. For instance, tyrosine aminotransferase, an enzyme of the neonatal cluster, can be evoked one or two days before birth by the administration of glucagon; however, administration of this hormone three or more days before birth cannot evoke the aminotransferase (4). Further experimentation demonstrated that cyclic adenosine monophosphate (cyclic AMP) can evoke tyrosine aminotransferase synthesis on the eighteenth or nineteenth day of gestation. Thus, the competence of the liver to produce cyclic AMP on the nineteenth day of gestation in response to glucagon may be the key to the early appearance of the aminotransferase.

Greengard has also done some work on the relationship of gene expression to enzymic differentiation. Unfortunately, the Jacob-Monod model of enzyme regulation in bacteria does not apply in higher organisms since there is no evidence of the existence of regulatory genes, operons, or repressors. She postulates that the active gene for synthesis of a specific enzyme is a necessary but not the only condition for

enzyme synthesis. Unless the messenger ribonucleic acid (mRNA) is present, the enzyme is not produced; however, the mRNA may be present hours or days before the production of the enzyme. Thus, the absence of the enzyme indicates only that at least one of the steps required for its synthesis is inoperative, but it does not indicate the identity of the inoperative step. Conversely, when an enzyme appears, all necessary reactions are operative, but it is not apparent which step has suddenly become operative. The final prerequisite might have been the formation of the mRNA, but it is also possible that transcription was operative earlier and that the appearance of some cofactor was the missing factor necessary for completion of the protein chain. Many researchers are attempting to implicate endocrine secretions as factors which may control protein synthesis at some point or may provide the cofactors necessary for the completion of protein chains.

The specific hormones administered in this study have been shown to affect the enzyme profile of the adult liver and thus may have a prenatal influence on enzymic differentiation. Insulin has been found to increase the activities of adult UDPG-glycogen glucosyltransferase, hexokinase, and glucokinase (29,30). Furthermore, insulin has been shown to be necessary for normal development of glucosyltransferase in fetal chick liver explants (15). Grillo has shown a correlation between formation of this enzyme and the initiation of

fetal insulin secretion (12). Insulin has also been shown to trigger early development of neonatal glucokinase (23).

Small doses of thyroxine potentiate the effects of insulin on glycogen synthesis and glucose utilization (29). Thyroxine is also known to increase adult hexokinase activity (31). Specific effects of growth hormone on the enzymes tested here are not documented in the literature; however, growth hormone is known to increase the release of insulin and to increase the levels of glucose-6-phosphate in the adult. Such evidence in the adult justifies the study of these hormones in the fetus, though it does not guarantee the same results.

## CHAPTER III

## MATERIALS AND METHODS

Surgical Procedures and Experimental DesignsGlycogen Experiment

Virgin Sprague-Dawley-Holtzman rats ranging in weight from 250 to 275 grams were bred and considered pregnant when spermatozoa were found in the vaginal tract; the day of spermatozoa detection was designated as gestational day zero. On the nineteenth or the twentieth day of gestation, an abdominal incision was made on the female (ether anesthesia) to expose the uterus. The fetuses were injected with 10  $\mu$ l. of either thyroxine ( $T_4$ ) (0.01  $\mu$ g.), bovine insulin (0.004 units), ovine growth hormone (GH) (0.01 units), or saline (0.9% sodium chloride). The dosages of hormones were determined by adjusting (to average fetal weight) those dosages considered to be physiological in the adult. All hormones were dissolved in saline. The saline-injected fetuses served as controls. Injections were intraperitoneal.

Fetuses within each horn of a given mother were alternately injected with saline or with a hormone. Care was taken to assure that each litter contained at least one fetus in each of the three hormone treatment groups. A control was obtained for each position in the horn within a given mother.

Following the injections, the abdominal muscle was sutured and the skin closed with wound clips. Twenty-four hours following the injection, the mother was anesthetized with ether and the fetuses were removed and weighed. The liver was then removed from each fetus, weighed, and assayed for glycogen.

Ether anesthesia is known to mobilize glycogen. However, this mobilization is a function of the initial concentration of glycogen. Therefore the effect would be to minimize the differences between the mean of the glycogen level in a treatment group and that in a control group. Thus, the use of ether anesthesia could obscure a true treatment effect. However, the presence of a significant difference between the mean of a treatment group and the mean of the control group would be indicative of a distinct effect by the hormone. A similar effect of minimizing the differences between groups may be seen on those enzymes assayed for in the following experiment. However, little is known of the effect of ether on enzyme activities.

#### Enzyme Experiment

The surgical procedure used in the experiments involving the effect of hormone administration on enzyme activity was similar to the procedure discussed above with the following changes. Pregnant Sprague-Dawley-Holtzman rats were laparotomized on either the fifteenth or the nineteenth day of gestation. Fetuses injected on the fifteenth day were

treated with five  $\mu$ l. of  $T_4$  (0.005  $\mu$ g.), insulin (0.002 units), GH (0.005 units), or saline (0.9%). Due to the small size of the livers, two or three livers from the same treatment group were pooled for assay. Fetuses injected on the nineteenth day of gestation were treated with the same hormones and the same dosages as those in the glycogen experiment.

In this experiment, one horn within a female was treated with saline while the other horn was treated with one hormone. Care was taken to assure alternation of the horn used for the controls from mother to mother. This design allowed comparison of a treatment group to its own set of controls.

Following removal, the liver tissues were quickly frozen in liquid nitrogen and stored frozen until assay. Each liver was assayed for the activities of the independent and dependent forms of UDPG-glycogen glucosyltransferase or for the activities of hexokinase and glucokinase.

#### The Isolation and Quantitation of Glycogen

Glycogen was determined by a modification of the method of Good, Somogyi, and Cramer (32). Livers from 20 and 21 day fetuses treated 24 hours previously with growth hormone, insulin, thyroxine, or saline were collected. Each liver was added to 1.5 ml. of 30% KOH and then heated for twenty minutes in a boiling water bath. Glycogen was precipitated with 2.5 ml. of 95% ethanol overnight at room temperature. The precipitates were collected after centrifugation for

ten minutes at 2500xg, and washed three times by centrifugation using 3 ml. of 95% ethanol at each step. After the final wash, the precipitates were air dried, and the samples were frozen until assay.

The glycogen samples were hydrolyzed in 2 ml. of 2 N H<sub>2</sub>SO<sub>4</sub> for 2 hr. at 100°C., diluted to 5 ml. with distilled water, and 0.05 ml. of each glycogen sample was added to 4 ml. of Glytel Reagent (Pfizer). A reagent blank using 0.05 ml. of distilled water and 4 glucose standards (50 mg. %, 100 mg. %, 200 mg. %, 400 mg. %) were prepared similarly. The reagent blank, glucose standards, and experimental samples were placed in a boiling water bath for exactly 10 min., cooled to room temperature, and the absorbance read at 630 nm. The glucose hydrolyzed from the glycogen reacts stoichiometrically with the ortho-toluidine in the Glytel Reagent to yield a colored complex.

A standard curve was prepared by plotting mg. % glycogen of the standards versus their respective absorbances. The absorbances of the unknown could then be converted to mg. % glycogen by interpolating from this curve and correcting for dilution.

#### UDPG-glycogen glucosyltransferase Assay

UDPG-glycogen glucosyltransferase activity was determined by the method of Das and Hem with some modification (33). The frozen tissue was homogenized with an ice-cold buffer

(pH 7.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM KF, and 10 mM Tris(hydroxymethyl)aminomethane hydrochloride in a Potter-Elvehjem homogenizer. The ratio of tissue to buffer was one gram to six ml. The homogenate was centrifuged at 4500xg for 10 min. The UDPG-glycogen glucosyltransferase assay mixture contained 6.7 mM UDP-glucose-<sup>14</sup>C (500,000 dpm/ $\mu$ m.) (New England Nuclear), 10 mg./ml. of rabbit liver glycogen, and 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (pH 7.8). The tissue extract (0.05 ml.) was added to tubes containing 0.1 ml. of the assay mixture; incubation was carried out at 30°C. for fifteen minutes in the presence and absence of glucose-6-phosphate (10 mM). The reaction was stopped by the addition of 1 ml. of 6% trichloroacetic acid (TCA) containing 1 mg./ml. glycogen and 2 mg./ml. LiBr. Glycogen was precipitated by the addition of 2 volumes (2.3 ml.) of 95% ethanol. The tubes were allowed to stand refrigerated overnight to assure complete precipitation. The precipitate was filtered through Gelman Metrical GA-6 filters with a pore size of 0.45  $\mu$ m. and washed six times with 2 ml. of 66% ethanol. The glycogen retained by the filter was hydrolyzed with 0.75 ml. of diazyme (amyloglucosidase, Sigma) in 1.0 M sodium acetate buffer (0.1 mg./ml.) at pH 5.0 in liquid scintillation vials. Fourteen ml. of Handifluor was added to the vials along with 0.35 ml of 1 N HCl to facilitate solubilization of the buffer in the scintillant. Radioactivity was measured in a Beckman LS-100C liquid scintillation counter.

An efficiency curve was prepared by plotting the external standardization ratios (ESR) against the percentage of efficiency of several matched radioactive samples to which had been added varying quantities of acetate buffer. The readings obtained from the scintillation counter were converted from counts per minute (cpm) to disintegrations per minute (dpm) by use of the following formula:

$$\frac{\text{cpm unknown} - \text{cpm blank}}{\text{efficiency}} = \text{dpm of UDP-glucose-}^{14}\text{C incorporated}$$

The reaction mixture contained  $2.05 \times 10^{-6}$   $\mu$ m. UDPG/dpm. In all cases the aliquot assayed contained 0.008 g. of liver tissue, and incubation was carried out for 15 minutes. The activity of UDPG-glycogen glucosyltransferase was calculated in the following way:

$$\frac{[(\text{sample dpm} \times 2.05 \times 10^{-6} \mu\text{moles UDPG/dpm}) \div 0.008 \text{ g.}] \div 15 \text{ min.}}{\mu\text{moles UDPG incorporated/g. liver/minute}}$$

#### Hexokinase and Glucokinase Assays

The activities of hexokinase and glucokinase were determined by a modification of the method of Jamdar and Greengard (21). The liver tissue was homogenized in five volumes of cold 0.15 M KCl. The homogenate was centrifuged at 145,000xg for 30 minutes; the supernatant was assayed for glucokinase

and hexokinase. The supernatant (0.025 ml.) was added to 3 ml. of an incubation medium containing a 50 mM triethanolamine, 5 mM EDTA, and 5 mM  $MgCl_2$  buffer, pH 7.6; 0.5 mM NADP; 5 mM ATP; 0.2 units of glucose-6-phosphate dehydrogenase (Sigma), and one of two concentrations of glucose, 100 mM or 0.5 mM. The formation of reduced NADP was measured by the increase in optical density at 340 nm. over a 10 min. incubation at 24°C. A Beckman DBG spectrophotometer was used to measure optical density. Hexokinase was determined from the difference between the rates of incubations with 0.5 mM glucose in the presence and absence of ATP. Glucokinase was determined from the difference in reaction rates between the incubation with 100 mM glucose and the incubation with 0.5 mM glucose. This method of determining glucokinase is valid because incubation with 100 mM glucose results in saturation of hexokinase (which has a lower  $K_m$  than glucokinase). All enzyme activity beyond the hexokinase activity in this assay can be attributed to glucokinase.

The rates in absorbance units/min. were converted to  $\mu m.$  of glucose phosphorylated/g. of liver/min. The extinction coefficient of NADP is 2 optical density units/ $\mu m.$  Each aliquot assayed contained 0.005 g. of liver tissue. The activities were calculated using the following formulas:

$$\frac{\text{reaction rate(units/min.)}}{2} = X \mu\text{moles NADP reduced/.005 g./min.}$$

$$= \frac{X}{2} \text{ } \mu\text{moles glucose phosphorylated}/.005 \text{ g./min.}$$

$$= 100(X) \text{ } \mu\text{moles glucose phosphorylated/g./min.}$$

Early experiments demonstrated that the hexokinase reaction rate was linear with time beyond ten minutes, indicating that rates measured over that time were indeed measurements of initial rates.

#### Statistical Methods

The data obtained from glycogen determinations were analyzed using Tukey's Honestly Significant Difference method (34); probability values from day 20 and day 21 were combined according to the method of R. A. Fisher to yield one probability (34).

The data concerning the effects of hormonal administration on enzyme activity were analyzed by means of a two-way analysis of variance using the Statistical Package for the Social Sciences. Where linear regression computations indicated a high correlation between fetal weight and enzyme activity, the analysis of variance included weight as a covariant.

The effect of hormone treatment on fetal weight was tested with one-way analysis of variance. The effect of hormone administration on placental weight was analyzed using Tukey's Honestly Significant Difference method.

## CHAPTER IV

## RESULTS

Glycogen determinations on fetal livers exposed to growth hormone, insulin, and thyroxine demonstrated a significant increase in glycogen accumulation with thyroxine administration as compared with saline administration ( $P \leq 0.01$ ) (See Table 1). Subsequent experiments on the effects of thyroxine, growth hormone, and insulin on the activity of the independent form of UDPG-glycogen glucosyltransferase yielded no treatment effect on that enzyme at either day 16 or day 20. The three hormones also had no effect on the activity of the dependent form of glucosyltransferase (See Table 2). Fetal glucokinase activity was found to be nonexistent on both day 16 and day 20 (See Table 3). Treatment with growth hormone was found to approximately double the hexokinase activity on days 16 and 20 (See Table 4 and Figures 1 and 2). While insulin-treated fetuses showed an upward trend in hepatic hexokinase activity when compared to controls within the same mother, there was no significant difference between the means (See Figure 3). Thyroxine administration produced no significant effect on hexokinase activity.

Only in analyzing the effect of insulin administration on hexokinase activity on day 20 was there found to be a high

correlation between fetal weight and enzyme activity; hence, this was the only case in which weight was used as a covariant in the statistical analysis.

Thyroxine administration effected an increase in fetal weight on day 20; the difference between the control and treated fetal weights was significant ( $P \leq 0.03$ ) (See Table 5). No other treatments altered fetal weights.

Differences between placental weights of the experimental and control groups were insignificant (See Table 6).

Table 1. The Effects of Hormone Administration on Fetal Glycogen on Day 20 and Day 21. (mg. glycogen/g. liver)

	Saline	Growth Hormone	Insulin	Thyroxine
Day 20 <sup>1</sup>	33.3 ± 4.52 (N = 7)	32.3 ± 4.51 (N = 15)	39.9 ± 3.63 (N = 13)	50.4 ± 1.67 <sup>2</sup> (N = 12)
Day 21 <sup>1</sup>	57.5 ± 3.99 (N = 27)	66.9 ± 5.14 (N = 13)	69.2 ± 4.69 (N = 12)	70.2 ± 4.73 <sup>2</sup> (N = 13)

<sup>1</sup> All data above is listed as mean ± standard error of the mean.

<sup>2</sup> Significantly different from saline controls ( $P \leq 0.01$ ) using the method of R. A. Fisher (34) to combine the probability values for both time periods (obtained using Tukey's Honestly Significant Difference method).

Table 2. The Effect of Hormone Administration on Fetal  
UDPG-glycogen glucosyltransferase I and D.  
( $\mu$ M/g. of liver/min.)

	Growth Hormone		Insulin		Thyroxine	
	I	D	I	D	I	D
Day 20 <sup>1</sup> Treatment	.052 $\pm$ .018 (N = 9)	.032 $\pm$ .011 (N = 9)	.123 $\pm$ .023 (N = 10)	.045 $\pm$ .015 (N = 10)	.082 $\pm$ .025 (N = 12)	.041 $\pm$ .014 (N = 12)
Control	.096 $\pm$ .026 (N = 9)	.040 $\pm$ .019 (N = 9)	.121 $\pm$ .040 (N = 7)	.042 $\pm$ .023 (N = 7)	.077 $\pm$ .017 (N = 14)	.051 $\pm$ .015 (N = 14)
Statistics <sup>2</sup>	P $\leq$ .265	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999
Day 16 <sup>1</sup> Treatment	.011 $\pm$ .002 (N = 7)	.014 $\pm$ .007 (N = 7)	.015 $\pm$ .005 (N = 7)	.022 $\pm$ .009 (N = 7)	.014 $\pm$ .003 (N = 5)	.017 $\pm$ .008 (N = 5)
Control	.014 $\pm$ .003 (N = 6)	.010 $\pm$ .005 (N = 6)	.016 $\pm$ .005 (N = 7)	.023 $\pm$ .011 (N = 7)	.005 $\pm$ .001 (N = 5)	.015 $\pm$ .007 (N = 5)
Statistics <sup>2</sup>	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999

<sup>1</sup> All data above is listed as mean  $\pm$  standard error of the mean.

<sup>2</sup> Analysis of variance.

Table 3. The Effect of Hormone Administration on Fetal Glucokinase Activity.  
( $\mu$ M/g. of liver/min.)

	Growth Hormone	Insulin	Thyroxine
Day 20 <sup>1</sup>			
Treatment	.019 $\pm$ .008 (N = 9)	.043 $\pm$ .024 (N = 8)	.011 $\pm$ .005 (N = 15)
Control	.032 $\pm$ .015 (N = 10)	.041 $\pm$ .019 (N = 9)	.019 $\pm$ .014 (N = 12)
Statistics <sup>2</sup>	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999
Day 16 <sup>1</sup>			
Treatment	.002 $\pm$ .002 (N = 10)	.007 $\pm$ .007 (N = 6)	.000 (N = 5)
Control	.003 $\pm$ .002 (N = 8)	.004 $\pm$ .002 (N = 7)	.017 $\pm$ .017 (N = 5)
Statistics <sup>2</sup>	P $\geq$ .999	P $\geq$ .999	P $\geq$ .999

<sup>1</sup> All data above is listed as mean  $\pm$  standard error of the mean.

<sup>2</sup> Analysis of variance.

Table 4. The Effect of Hormone Administration on Fetal Hexokinase Activity.  
( $\mu\text{M/g. of liver/ min.}$ )

	Growth Hormone	Insulin	Thyroxine
Day 20 <sup>1</sup>			
Treatment	.318 $\pm$ .041 (N = 9)	.258 $\pm$ .045 (N = 8)	.140 $\pm$ .021 (N = 15)
Control	.163 $\pm$ .029 (N = 10)	.180 $\pm$ .038 (N = 9)	.168 $\pm$ .032 (N = 12)
Statistics <sup>2</sup>	P $\leq$ .020	P $\leq$ .125	P $\geq$ .999
Day 16 <sup>1</sup>			
Treatment	.091 $\pm$ .033 (N = 10)	.061 $\pm$ .011 (N = 6)	.058 $\pm$ .017 (N = 5)
Control	.026 $\pm$ .013 (N = 8)	.051 $\pm$ .021 (N = 7)	.046 $\pm$ .024 (N = 5)
Statistics <sup>2</sup>	P $\leq$ .023	P $\geq$ .999	P $\geq$ .999

<sup>1</sup> All data above is listed as mean  $\pm$  standard error of the mean.

<sup>2</sup> Analysis of variance.

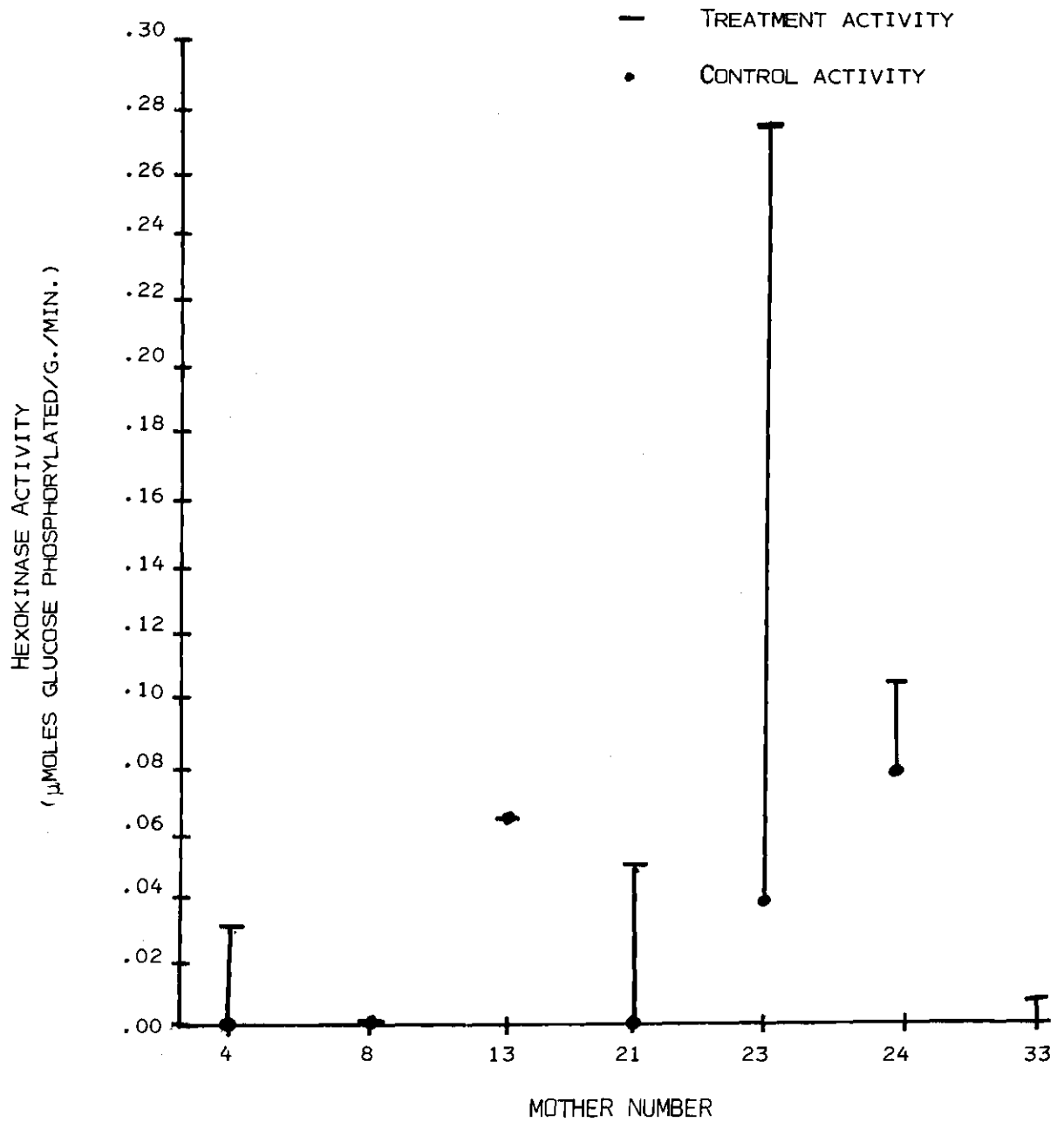


FIGURE 1. THE EFFECT OF GROWTH HORMONE ADMINISTRATION ON HEXOKINASE ACTIVITY AS COMPARED WITH SALINE ADMINISTRATION ON DAY 16 IN FETUSES OF THE SAME MOTHER.

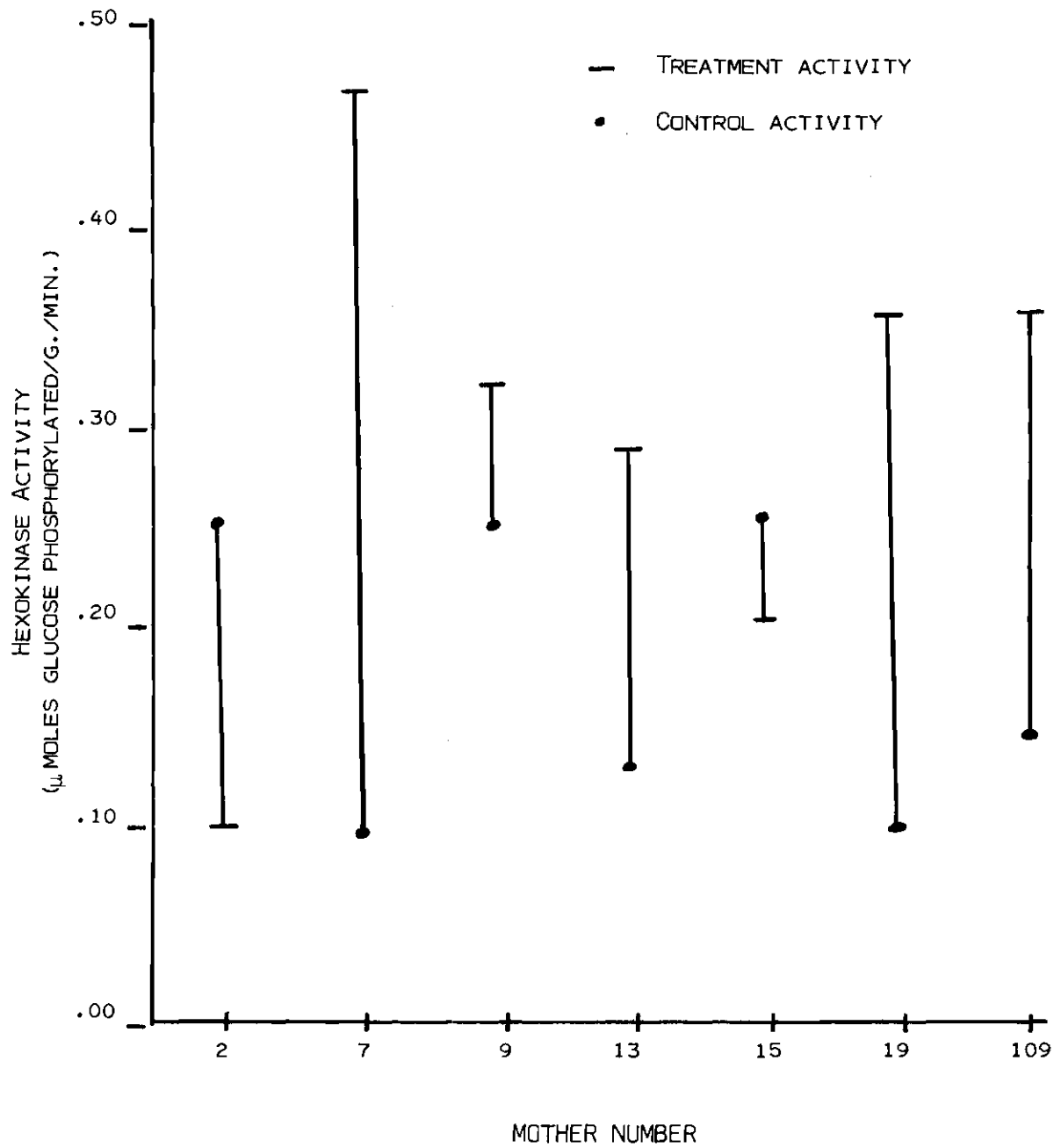


FIGURE 2. THE EFFECT OF GROWTH HORMONE ADMINISTRATION ON HEXOKINASE ACTIVITY AS COMPARED WITH SALINE ADMINISTRATION ON DAY 20 IN FETUSES OF THE SAME MOTHER.

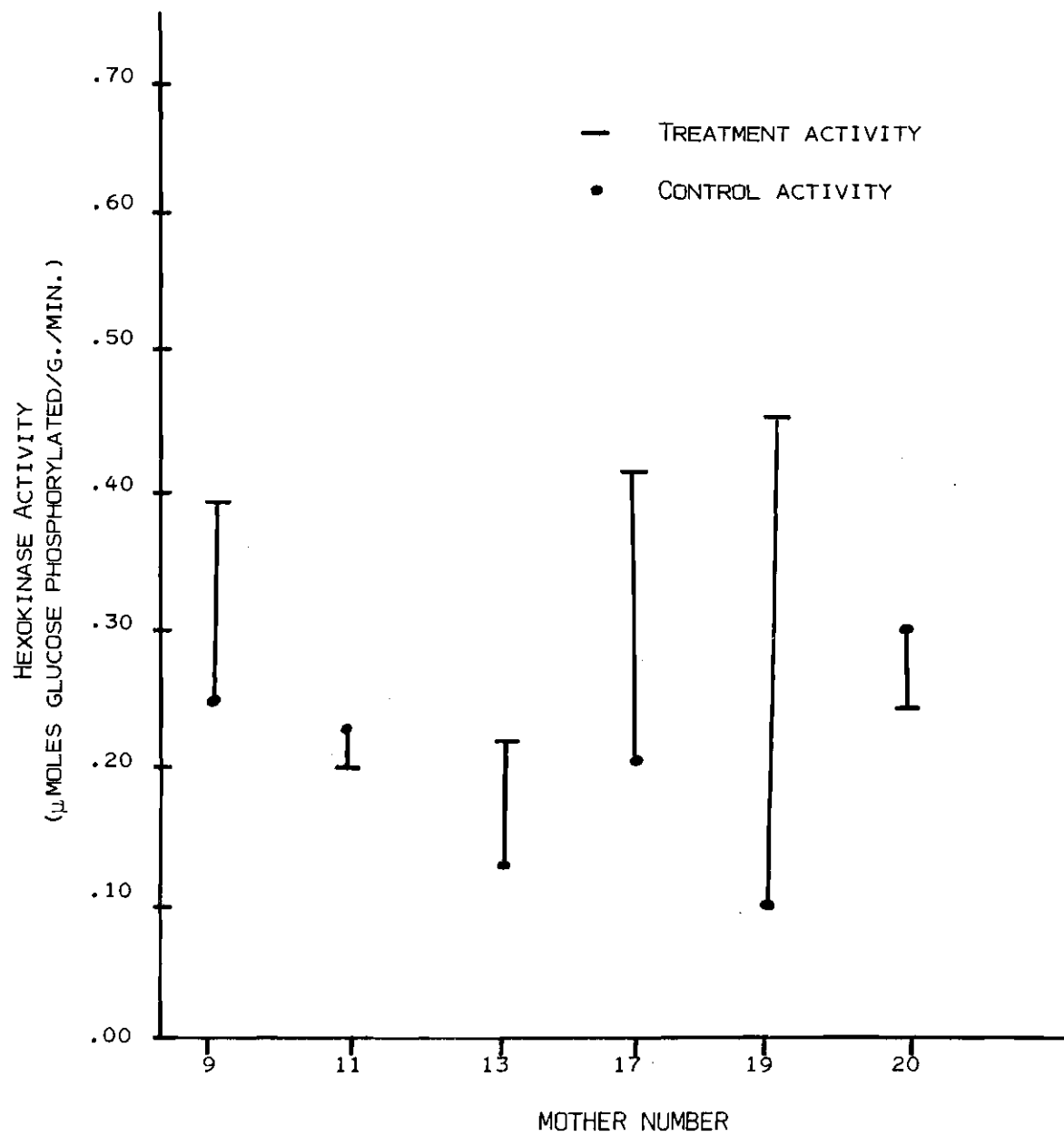


FIGURE 3. THE EFFECT OF INSULIN ADMINISTRATION ON HEXOKINASE ACTIVITY AS COMPARED WITH SALINE ADMINISTRATION ON DAY 20 IN FETUSES OF THE SAME MOTHER.

Table 5. The Effect of Hormone Administration on Fetal Weight.  
(grams)

	Growth Hormone	Insulin	Thyroxine
Day 20 <sup>1</sup>			
Treatment	3.83 ± .12 (N = 9)	3.16 ± .09 (N = 10)	3.79 ± .18 (N = 12)
Control	3.56 ± .13 (N = 10)	3.35 ± .17 (N = 7)	3.20 ± .18 (N = 14)
Statistics <sup>2</sup>	P ≤ .141	P ≤ .274	P ≤ .030
Day 16 <sup>1</sup>			
Treatment	.47 ± .06 (N = 10)	.47 ± .05 (N = 7)	.41 ± .04 (N = 5)
Control	.54 ± .08 (N = 8)	.42 ± .05 (N = 7)	.41 ± .05 (N = 5)
Statistics <sup>2</sup>	P ≤ .506	P ≤ .447	P ≤ .982

<sup>1</sup> All data above is listed as mean ± standard error of the mean.

<sup>2</sup> Analysis of variance.

Table 6. The Effects of Hormone Administration on Placental Weights of Fetuses at Day 20.<sup>1</sup>

	Saline	Growth Hormone	Insulin	Thyroxine
Placental weight <sup>2</sup> (grams)	0.605 ± .012 (N = 49)	0.612 ± .027 (N = 27)	0.591 ± .015 (N = 30)	0.599 ± .015 (N = 27)

<sup>1</sup> Treatment with Tukey's Honestly Significant Difference method showed no significant differences between treatment groups and controls.

<sup>2</sup> All data above is listed as mean ± standard error of the mean.

## CHAPTER V

## DISCUSSION

The majority of research data available on the effects of hormone administration in the fetus deals with the effects of glucocorticoids on glycogen metabolism and of thyroxine on some specific hormones of the late fetal and neonatal periods. A search of the literature yields a limited amount of reports on the effects of growth hormone or insulin administration in the fetus. Little is known about the effects of hormone administration on fetal metabolism; therefore, the available information on hormonal effects and mechanisms in the fetus is minimal. Because many aspects of fetal metabolism differ from that seen in the adult, it cannot be assumed that the administration of a particular hormone will cause the same effects in the fetus as in the adult. Furthermore, differences in hepatic competence in the fetus and in the adult make it difficult to assume that the effects of hormonal administration are mediated through identical mechanisms.

The Effects of Hormone Administration on Hepatic UDPG-glycogen glucosyltransferase

Hepatic glycogen determinations on fetuses treated with hormones indicated a significant effect of thyroxine on glycogen deposition ( $P \leq 0.01$ ). These results served as an

impetus for testing the effect of thyroxine administration on UDPG-glycogen glucosyltransferase since this enzyme controls the deposition of fetal glycogen. The effects of growth hormone and insulin administration were also tested because these hormones are known to produce glycogen deposition in the adult.

UDPG-glycogen glucosyltransferase exists in two forms in liver (35). The form which is active in the absence of glucose-6-phosphate is glucosyltransferase I (independent); the form active only in the presence of glucose-6-phosphate is glucosyltransferase D (dependent). It is generally believed that glucosyltransferase I is the physiologically active form in heart, muscle, and liver tissue. The D form is considered to be physiologically inactive because of its low affinity (high  $K_m$ ) for glucose-6-phosphate and because of inhibition by inorganic phosphate and adenosine triphosphate. Glucosyltransferase I is active because it has a high affinity for glucose-6-phosphate and lower  $K_m$  for the substrate uridine diphosphoglucose (36).

It has already been noted that the earliest detection of UDPG-glycogen glucosyltransferase corresponds with the onset of insulin secretion on the twelfth day of gestation and that fetal glycogen deposition is enhanced by glucocorticoid administration.

In these experiments, hormone administration resulted in no significant effect on the activity of either the I or

the D form of glucosyltransferase. The effect of thyroxine on glycogen deposition might be achieved by increased substrate availability for the reaction. For example, thyroxine treatment in adults can elevate blood glucose levels thereby increasing incorporation into glycogen (37). The effect in the fetus could be achieved by an increase in the permeability of the hepatocyte to glucose or by an increase in activity of one or both of the two enzymes which catalyze the steps immediately before the one catalyzed by glucosyltransferase in the glycogenic pathway. The result of an increase in either of these enzymes (phosphoglucomutase and UDPG pyrophosphorylase) would increase the availability of UDPG.

Another possible explanation for the effect of thyroxine on glycogenesis involves alterations of cellular metabolites other than glucose. Blatt et al. found that altering levels of metabolites (such as glucose-6-phosphate and adenosine triphosphate) can stimulate glycogen synthesis in the absence of glucosyltransferase activation; manipulation of intracellular metabolites can also regulate the activity of glucosyltransferase in the absence of hormonal influences (38). Under normal conditions changes in other metabolites or minerals (eg. magnesium, inorganic phosphate, nucleotides, sodium, potassium) may also serve a regulatory function. The administration of thyroxine is known to increase serum calcium levels and can accelerate active transport of sodium across isolated skin and bladder membranes (39, 40). Since the effect

of thyroxine on mineral metabolism in the hepatocyte may differ from that seen in the serum or in isolated membranes, further experiments should be undertaken to identify the effect of thyroxine on those hepatic metabolites which can regulate the activity of this enzyme. Identification of those substances which affect the levels of these metabolites could provide more insight into the regulation of glycogenesis.

The absence of a significant increase in glucosyltransferase activity with insulin treatment was surprising. Grillo's demonstration of the detection of glucosyltransferase concurrently with the onset of insulin secretion on the twelfth day of gestation indicated a possible interaction between the two. Since the effect of insulin on glucosyltransferase in the adult is mediated through the cyclic AMP system, the results obtained here may indicate lack of competence by the liver to respond to the hormone with cyclic AMP.

#### The Effects of Hormone Administration on Hepatic Hexokinase

The high activity of hexokinase before birth suggests a high capacity in liver for the phosphorylation of glucose. In the fetal liver, hexokinase is the rate-limiting enzyme in glycolysis (41). Although little is known about its hormone sensitivity in the fetus, hexokinase activity is affected by hormone administration in the adult. Thyroxine and insulin both increase hexokinase activity (42).

Since hexokinase activity is already high by the time

it is first measured (sixteenth day of gestation) in this experiment, the objective of the research was not to find a hormonal trigger for this enzyme but to obtain information concerning the hormonal control of hexokinase in the fetus.

Hexokinase exists as four isozymes in rat liver, which differ in kinetic properties and time of appearance. Isozyme A is the predominant isozyme in fetal rat liver with low levels of isozymes B and C; these three isozymes have low  $K_m$ s and reach their adult values by the time of weaning. At this time, the high- $K_m$  isozyme D (glucokinase) appears and reaches adult levels on the thirtieth postnatal day. While the amount of glucose intake seems to be a determinant of glucokinase activity at the time of weaning, very little is known about the differentiation of the other isozymes (43).

It is known that 85% of fetal hexokinase is in the form of isozyme A. The absence of glucokinase in the fetus is probably unimportant since control of fetal blood glucose concentration is maintained by the maternal liver. During early extrauterine life, the newborn rat can control its own carbohydrate metabolism without possessing hepatic glucokinase. Walker and Holland have attempted to imply that hepatic glucokinase may be of limited importance in glucose utilization and acts primarily as an overflow mechanism to handle glucose phosphorylation when blood glucose concentration increases above normal (44). Walker and Rao have emphasized that hepatic glucokinase activities measured in vitro with 100 mM glucose

as the substrate are never likely to be achieved in vivo where blood glucose concentration in the hepatic portal vein rarely rises above 11 mM (45). This confined role of glucokinase supports the concept that, under normal conditions, the role of the liver is to make glucose rather than to use it.

The slow rate of development of glucokinase, the steady hexokinase activities during glucokinase development, and the effects of known inhibitors of protein synthesis suggest that the development of glucokinase results from an initiation of and an increasing ability to synthesize new protein (44). Starvation during the time when glucokinase activity should be increasing results in a decrease in the observed glucokinase activity. Subsequent glucose feeding results in a rapid increase in glucokinase activity, indicating that the potential ability to synthesize glucokinase has increased despite the starvation. Thus, the presence of dietary glucose is not the only requirement for the development of glucokinase. Insulin can increase glucokinase activity primarily by its protein anabolic effect (45). Insulin may not only have an effect at the transcription level but also at the ribosomal level (46).

Although the low  $K_m$  hexokinases develop much earlier than glucokinase, perhaps the prenatal regulation of the four may be similar. Although no significant effect of insulin or thyroxine was seen on hepatic hexokinase activity, a distinct effect of growth hormone on both day 16 and day 20 was demon-

strated ( $P \leq 0.05$ ). Growth hormone promotes protein anabolism, and is known to be present in high plasma levels by day 19 of gestation (47). Perhaps the almost doubled activity of hexokinase in livers treated with growth hormone as compared to saline-treated fetal rats may be the partial result of this protein anabolic effect. Root suggested that human growth hormone may increase the ability of the ribosomes to attach to or to translate messenger ribonucleic acid (48). There is evidence in the adult that growth hormone stimulates amino acid transport across the cell membrane and into the cytoplasm of cells and that it stimulates amino acid incorporation into protein. This latter action of growth hormone is blocked by inhibitors of ribonucleic acid and protein synthesis (42). There is evidence that the activities of other fetal enzymes, such as glucose-6-phosphatase, may be increased by growth hormone administration in the fetus (Porterfield, unpublished observations). Thus, the effect of growth hormone administration on hexokinase may be the result of a protein anabolic effect resulting in increased production of several enzymes in the fetus. If the protein anabolic effect of growth hormone were a nonspecific one, one would have expected glucosyltransferase to be increased by this hormone. However, this effect was not demonstrated by this researcher.

In the adult, proteins made under the influence of growth hormone appear to increase the levels of cyclic AMP in cells (42), which results in a decrease in the activity of

hexokinase. However, these experimental results showed an increased hexokinase activity with growth hormone treatment, indicating perhaps that fetal liver on the nineteenth day of gestation may not possess the ability to produce adequate amounts of cyclic AMP in response to growth hormone or proteins influenced by growth hormone. Since the competence of the liver to form cyclic AMP in response to growth hormone has not been adequately studied in the fetus, we cannot be sure that the mechanism for fetal response to growth hormone is identical to that seen in the adult; however, should the two mechanisms be similar, the inability of the liver to form cyclic AMP in this situation combined with the protein anabolic effect seen with growth hormone could explain the noted increase in hexokinase activity.

Even though the hexokinase activity found with insulin administration was not statistically different from the control values obtained, there appears to be a definite upward trend in several of the hexokinase activities of insulin-treated fetal livers as compared to saline-treated livers within the same mother (See Figure 4). Use of a ranking statistic on the means of the treatment and control groups within mothers supported this bias, indicating a significant difference between the means ( $P \leq 0.02$ ). Experiments with a greater number of animals are needed in order to confirm this result.

Although thyroxine increases hexokinase activity in the adult, the same effect could not be demonstrated in the fetus.

Here, again, the liver may not possess the competence to respond to thyroxine with the mechanisms required for increased hexokinase activity on the days tested. Injection of thyroxine later in gestation or addition of possible missing cofactors concomitant with thyroxine administration might lead to a response in the fetus similar to that in the adult.

The increase in fetal weight noted with thyroxine administration might be associated with the protein anabolic effect of this hormone. This anabolic effect of thyroxine is achieved in the adult by an increase in the transfer of amino acids to the protein synthesizing site, thereby facilitating the incorporation of amino acids into protein.

## CHAPTER VI

## CONCLUSIONS

Thyroxine administration late in gestation results in increased glycogen deposition in fetal liver. However, thyroxine had no effect on the activity of either the I or the D form of fetal hepatic UDPG-glycogen glucosyltransferase on the sixteenth or the twentieth day of gestation. No effects on glucosyltransferase activity were obtained with growth hormone or insulin administration.

The increase in glycogen deposition following thyroxine administration does not appear to be mediated through increased activity of glucosyltransferase.

Glucokinase activity was found to be absent on the sixteenth and the twentieth days of gestation as expected.

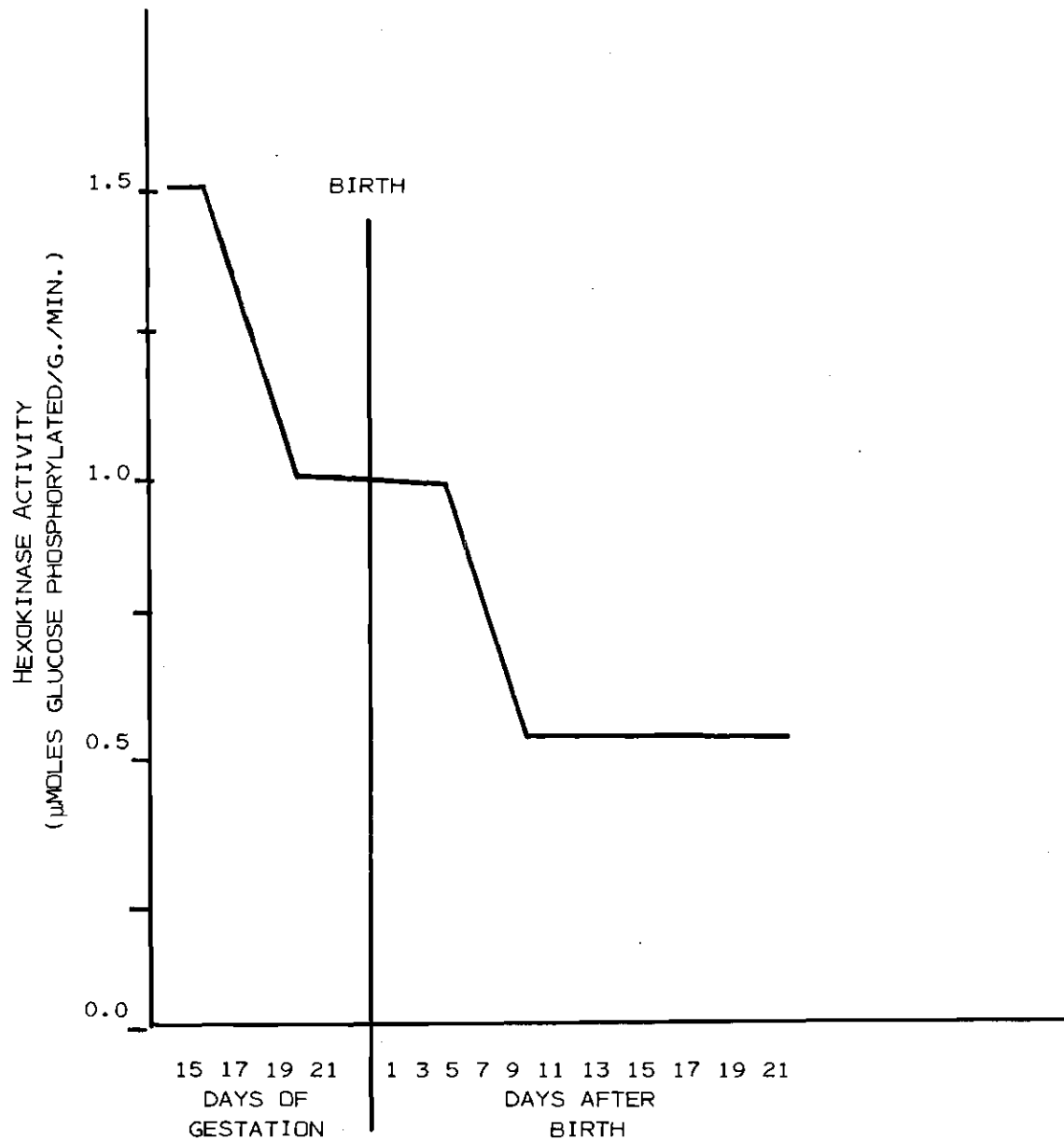
Hexokinase activity was increased significantly at day 16 and day 20 by the injection of growth hormone. This increase may be mediated through a protein anabolic effect of growth hormone.

## APPENDIX



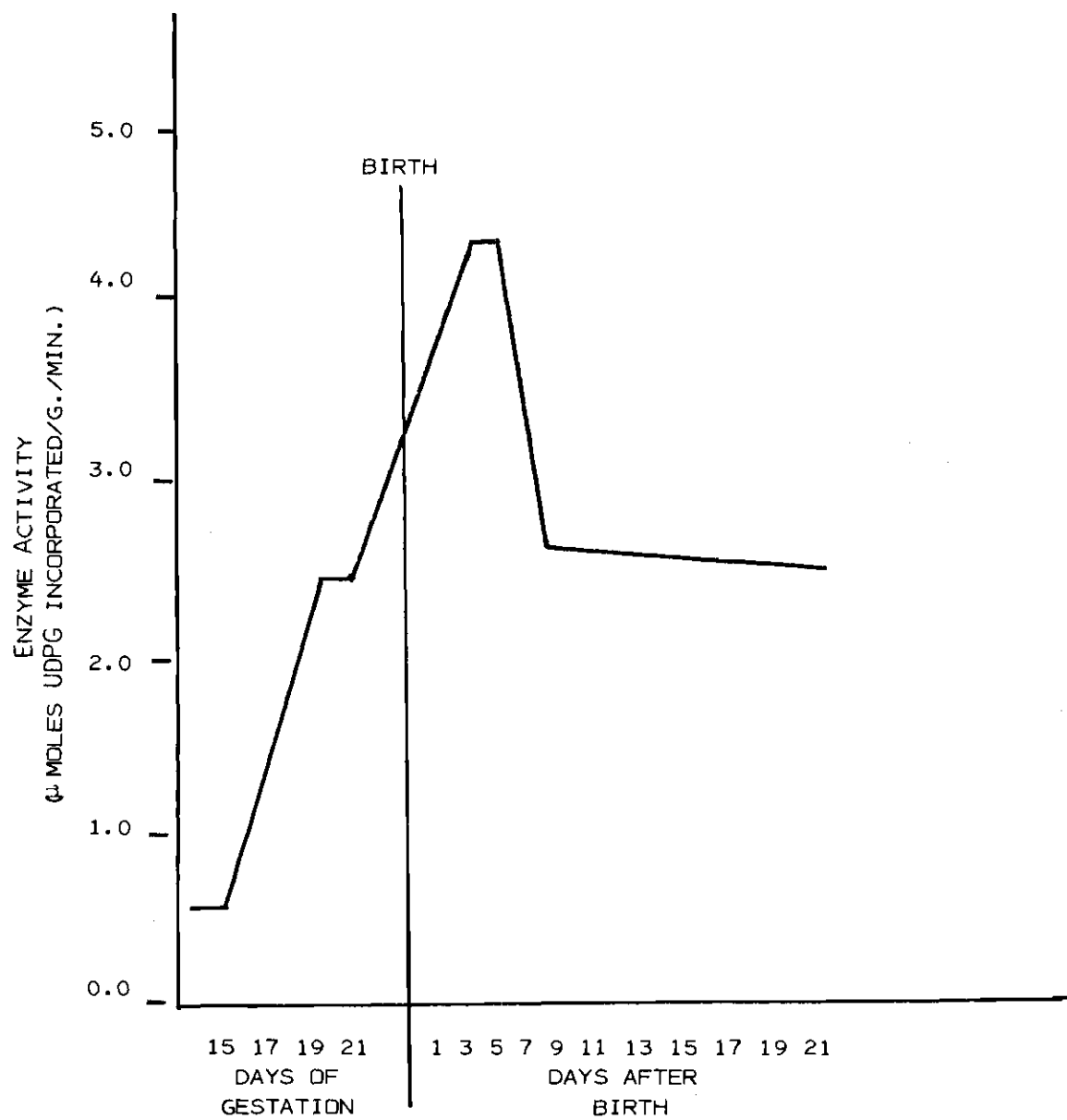
1. hexokinase or glucokinase
2. Glucose 6-phosphatase
3. phosphoglucomutase
4. UDPG pyrophosphorylase
5. Glycogen Synthetase
6. Glycogen phosphorylase
7. Glucose 6-phosphate dehydrogenase
8. 6-phosphogluconate dehydrogenase
9. phosphohexose isomerase
10. phosphofructokinase
11. fructose diphosphatase
12. Aldolase
13. Triosephosphate isomerase
14. Phosphoglyceraldehyde dehydrogenase
15. phosphoglycerate kinase
16. phosphoglycerate mutase
17. enolase
18. pyruvate kinase
19. pyruvate dehydrogenase
20. citrate condensing enzyme
21. citrate cleavage enzyme
22. malate dehydrogenase
23. malic enzyme
24. pyruvate carboxylase
25. (PEP) carboxykinase  
phosphoenolpyruvate carboxykinase

## APPENDIX B



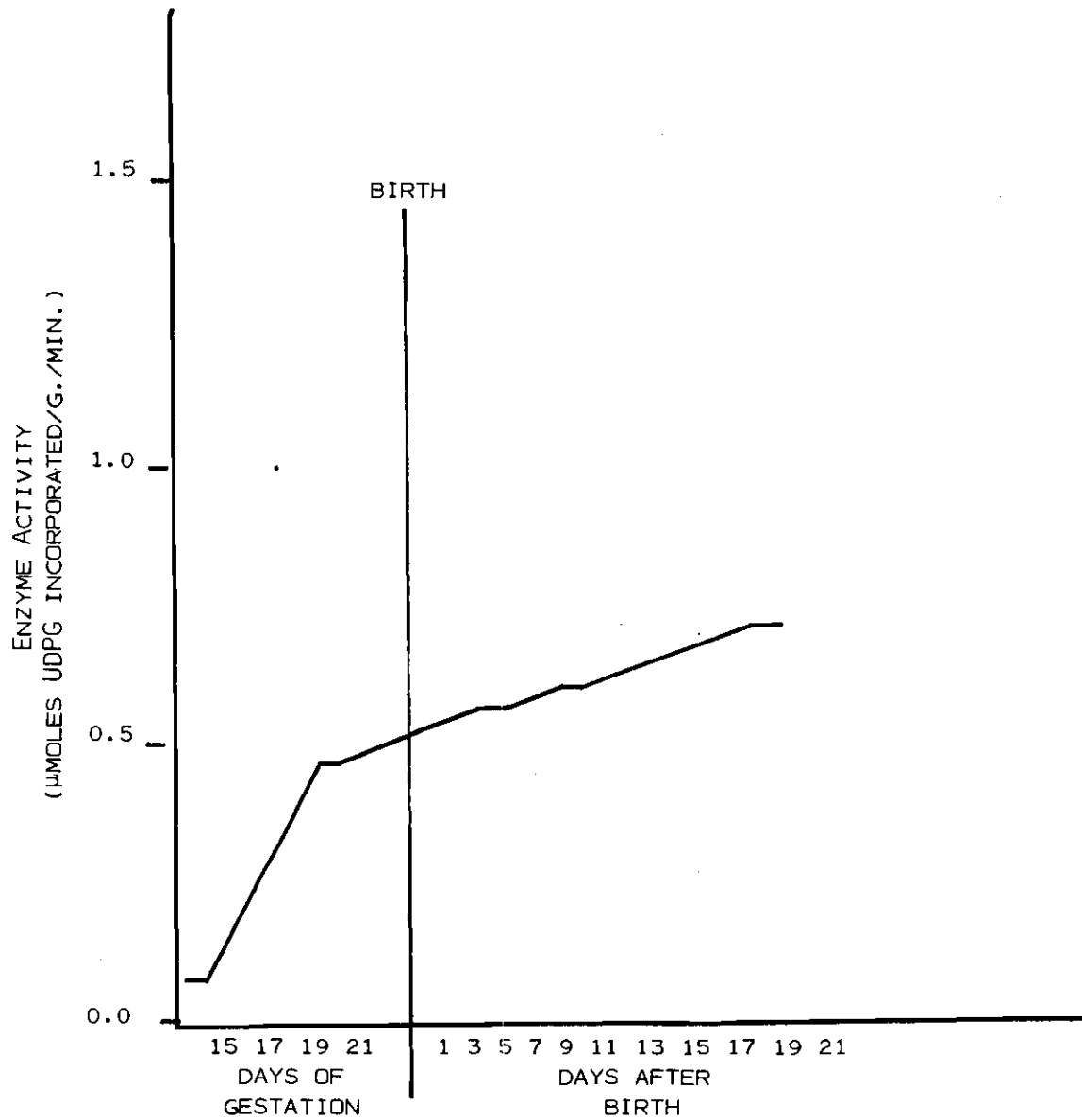
PROGRESSIVE DEVELOPMENTAL CHANGES OF HEXOKINASE ACTIVITY.  
(BASED ON SCHAUB ET AL., HORM. MET. RES., 4: 114, 1972)

## APPENDIX C



PROGRESSIVE DEVELOPMENTAL CHANGES IN TOTAL  
UDPG-GLYCOGEN GLUCOSYLTRANSFERASE (I+D).  
(BASED ON SCHAUB ET AL., HORM. MET. RES., 4: 115. 1972)

## APPENDIX D



PROGRESSIVE DEVELOPMENTAL CHANGES IN INDEPENDENT (I)  
UDPG-GLYCOGEN GLUCOSYLTRANSFERASE.  
(BASED ON SCHAUB ET AL., HORM. MET. RES., 4: 115, 1972)

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