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MATERNAL DIETARY GLUCOSE RESTRICTION AND ITS EFFECT ON AMNIOTIC FLUID AMINO ACID COMPOSITION

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ABSTRACT

Since glucose is an essential nutrient for normal fetal growth and development, the impact of reduced maternal dietary glucose supply, on amniotic fluid (amf) amino acid composition was investigated. Furthermore, this study investigated whether any resulting changes in the concentrations of amf amino acids could be predictive of fetal growth and metabolic status. Pregnant rat dams were fed isocaloric diets containing graded levels of dietary glucose (0, 12, 24 and 60%) and the amf amino acid content was analysed on gestational days (gd) 18.5 to 21.5. Carbohydrate restriction produced significant increases in the concentrations of amf isoleucine (on gd 21.5), tryptophan (on gd 18.5 and 21.5) and 3-methylhistidine (on gd 20.5 and 21.5). An interaction between diet and day of gestation modified amf taurine levels such that dams fed low carbohydrate diets showed significant increases in amf taurine as pregnancy progressed. Specific amf amino acids correlated with fetal growth parameters and fetal tissue glycogen reserves indicating the ability of amf composition to reflect fetal distress under conditions of compromised maternal nutritional status. A greater statistical predictability of amf constituents was obtained with fetal growth parameters than with fetal tissue glycogen reserves. These results suggest that amf amino acids are better predictors of fetal growth status than of fetal metabolic status.

RÉSUMÉ

Nous avons étudié l'impact d'une réduction de l'apport en glucose de la diète maternelle, le glucose étant un nutriment essentiel pour assurer une croissance et un développement normal du foetus, sur la composition en acides aminés du liquide amniotique (lam). De plus, cette étude s'est intéressée à l'éventualité qu'un changement de la concentration en acides aminés du lam pourrait servir d'indicateur de la croissance foetale et du statut métabolique. Des diètes de différentes teneur en glucose (0, 12, 24, et 60%) ont été offertes à des rats femelles enceintes et le contenu en acides aminées du lam a été analysé du jour 18.5 au jour 21.5 de la gestation. Une limitation de l'apport en hydrates de carbone a résulté en une augmentation significative de l'isoleucine (au jour de gestation 21.5), du trypophane (aux jours de gestation 18.5 et 21.5) et de la 3-méthylhistidine (aux jours de gestation 20.5 et 21.5) dans le lam. Une interaction entre la diète et le jour de gestation a modifié les niveaux de taurine dans le lam de sorte que les rates nourries aux diètes faibles en hydrates de carbone ont présenté une augmentation significative de la taurine du lam à mesure que la gestation progressait. Des acides aminés spécifiques du lam ont été corrélés avec des paramètres de la croissance foetale et avec les réserves en glycogène des tissus, démontrant ainsi l'habilité de la composition du lam en acides aminés à refléter une détresse foetale lorsque le statut nutritionnel maternel est compromis. Les constituants du lam ont permis d'obtenir une meilleure prédiction des paramètres de la croissance foetale que les réserves en glycogène des tissus. Ces

résultats suggèrent que les acides aminés du lam sont de meilleurs indicateurs de la croissance foetale que du statut métabolique foetal.

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OVERVIEW

Glucose is the principal metabolic fuel used by the developing fetus (Koski and Hill, 1990). When exogenous glucose supply to the fetus is curtailed, the reliance on alternative fuel sources, such as amino acids, is increased. The metabolic adaptations made by both the mother and fetus in response to compromised maternal nutritional status has been shown to be reflected in changes in the concentrations of maternal plasma amino acids, but these changes in the profile of plasma amino acids are confined to specific amino acids. Maternal energy restriction has been associated with decreased maternal plasma alanine concentrations and increased plasma branched-chain amino acids (BCAA), valine, leucine and isoleucine (Felig et al, 1972; Lemons and Schreiner, 1984).

These finding of altered plasma amino acids as a result of compromised maternal nutritional status are not dissimilar of what has been observed in the amniotic fluid (amf) compartment. A limited number of studies have examined the impact of maternal nutritional status on the composition of amf constituents. One of the first studies to investigate the effects of maternal fasting on amf amino acid concentrations, showed that maternal fasting resulted in significant decreases in amf alanine and marked increases in amf BCAA (Felig et al, 1972). Recently, in a glucose restricted model which induced intrauterine growth retardation (IUGR), IUGR was associated with decreased amf glucose concentrations and increased amf uric acid concentrations (Koski and Fergusson, 1992).

Attempts have been made to correlate plasma and amf constituents with fetal maturity. Positive correlations were found with maternal plasma glycine and lysine and negative correlations with plasma histidine and fetal age (Moghissi et al, 1975). Negative correlations also were found with amf alanine, histidine, taurine, phenylalanine, tyrosine, proline and the BCAAs and fetal age (Mesavage et al, 1985). It has been suggested that the amf amino acid profile is a more accurate mirror of fetal status than the maternal plasma amino acid profile. This stems from the observation that little correlation was found to exist between maternal plasma amino acid concentrations with amf amino acid concentrations, however correlations were found with a number of amf amino acids and amino acid levels in fetal urine (Cockburn et al, 1970; A'Zary et al, 1973).

No attempts have been made to correlate changes in the composition of amf amino acids with fetal growth and metabolic status and maternal diet. The consequences of reduced maternal dietary glucose supply, glucose being an essential nutrient for normal fetal growth and development (Koski and Hill, 1990) on amf composition, specifically the amino acid composition, has not been investigated even though IUGR and increased perinatal morbidity and mortality has been observed in the offspring of glucose restricted dams (Koski and Hill, 1990; Koski and Fergusson, 1992; Lanoue, 1993) and bitches (Romsos, 1981). The purpose of this study, therefore, was to investigate the effects of maternal dietary glucose restriction on amf amino acids and to determine whether any resulting changes in the concentrations of amf amino acids could be predictive of fetal growth and metabolic status.

The following chapter provides information on the profile of amf and plasma constituents, with greater focus on the amino acid composition, during altered maternal nutritional status. In addition, it summarises present knowledge on the use of amf and plasma constituents to diagnose fetal maturity and IUGR.

CHAPTER 1 - LITERATURE REVIEW

I. AMNIOTIC FLUID COMPOSITION

There is considerable interest in determining whether amniotic fluid (amf) composition, particularly the concentrations of glucose and amino acids in amf, reflects the metabolic milieu of the developing fetus. In spite of this interest, questions regarding the source, turnover rate and fate of glucose and amino acids in amf still remain unresolved. This difficulty stems from the fact that the mother, fetoplacental unit and amf are a complex and intimately related three-compartment system (Dooley et al, 1982). The contribution that each of these compartments makes to the composition of amf substrate levels varies with fetal age. In early gestation, the amf composition is very similar to maternal and fetal extra-cellular fluids due to the high permeability of fetal membranes to sodium and water (Lind, 1981). In contrast, in later pregnancy, amf osmolarity and sodium and glucose concentrations decrease while urea and creatinine concentrations increase (Lind, 1981). These changes in the composition of the amf have been attributed to the keratinization of fetal skin and to the maturation of fetal renal functions (Brace, 1989; Queenan, 1990). Amf becomes a repository for a number of secretions and excretions from the fetal respiratory tract, intestinal tract, umbilical cord, salivary glands and fetal urine, with fetal urine being the predominant source of amf with advancing gestational age (Mandelbaun and Evans, 1969; Pueschel et al, 1986). Therefore, as gestation progresses, the amf resembles less that of maternal and fetal serum and approaches that of fetal urine (Brace, 1989).

A. Profile of amniotic fluid constituents

The profile of amf constituents during pregnancy has contributed to the present understanding of the origin and fate of amf constituents, particularly glucose and amino acids. Amf glucose concentrations have been observed to decrease with the progression of pregnancy. The concentration of amf glucose is high early in human pregnancy (3.6mmol/L), falling to half this value at 20-32 weeks and continues to decrease to very low levels at term (0.7mmol/L) (Drazancic and Kuvacic, 1974). Generally in human studies, the concentrations of some amino acids in amf also have been shown to diminish with the progression of pregnancy (Emery and Scrimgeour, 1970; O'Neill et al, 1971; Scott et al, 1972; Dallaire et al, 1974; Velazquez et al, 1976). The essential amino acids show a more pronounced decrease than the non-essential amino acids. Specifically, Emery and Scrimgeour (1970) studied amino acid concentrations in amf from the 9^{th} to the 40^{th} week of pregnancy. The authors found that serine, glycine, phenylalanine, lysine and arginine progressively decreased during this period while no significant change occurred throughout gestation with cysteic acid, phosphoethanolamine, ethanolamine and proline. In a separate study (Dallaire et al, 1974), phenylalanine, tyrosine, valine, leucine, isoleucine, lysine, alanine and histidine decreased from the 10^{th} to the 40^{th} week of gestation. No significant change occurred with glycine, serine, threonine, aspartate, asparagine, glutamate, arginine, methionine, proline, ornithine, anserine, taurine and citrulline during this same period (Dallaire et al, 1974). In contrast, however, some amf amino acids have been shown to increase with the progression of pregnancy, namely ethanolamine, taurine (O'Neill et al, 1971),

cysteic acid (Emery and Scrimgeour, 1970; Kang and Scanlon, 1974) and glutamine (Mesavage et al, 1985).

The physiologic mechanisms responsible for diminishing amf glucose and amino acid concentrations with advancing gestational age in human pregnancy has not been completely elucidated. Factors that may be involved in the changes in the concentration of amf glucose and amino acids observed during gestation are briefly described below. Any or all of these three factors may be involved in the effect that gestational age has on amf glucose and amino acid concentrations in humans:

1) ***Kidney maturation:*** The observation that the concentration of glucose and amino acids decrease in the fetal urine as pregnancy progresses (Cockburn et al, 1970; Velazquez et al, 1976) suggests maturation of fetal renal tubular function, that is, greater renal tubular selectivity of substrates which begins around the 10th to 12th week of gestation (A'Zary et al, 1973; Dooley et al, 1982; Mesavage et al, 1985) is responsible. It is also noted that amf is initially isotonic, i.e., similar to maternal and fetal extra-cellular fluids, but becomes hypotonic with increasing output of fetal urine. This dilution factor could explain the general decrease in total glucose and amino acid concentrations towards term and may also explain the increase in the urea and creatinine concentrations, resulting from the maturation of the urinary system (Pueschel et al, 1986). Amf is particularly rich in taurine, histidine and ethanolamine, which reportedly occur in high concentrations in fetal urine (Cockburn et al, 1970; Emery and Scrimgeour, 1970; O'Neill et al, 1971; Velazquez et al, 1976). These observations further illustrate the close relationship

that exists between amf and fetal urine, that is, fetal urine makes a significant contribution to the make-up of amf constituents.

2) ***Maturation of gastrointestinal tract:*** Secondly, maturation of the fetal gastrointestinal function may increase the clearance of amf glucose and amino acids by means of fetal swallowing and gut absorption (Dooley et al, 1982).

3) ***Changes in placental diffusion:*** Glucose homeostasis is also influenced by the amnion and the placenta which both are known to actively metabolise glucose. In addition, the maturing placenta has been shown to have a high rate of glycolysis (Pueschel et al, 1986).

The amino acid concentration in amf is species specific but currently it is not yet known why there are differences among species in the concentrations of amf amino acids. In rats, however, the amino acid concentrations in amf increases with advancing gestational age (normal length of gestation for rats is 21-22 days) (Wirtschafter, 1958; McEvoy-Bowe et al, 1987). The total free amino acid concentration in amf was shown to increase from day 13 to day 20 of gestation (Wirtschafter, 1958). The authors reported increases in the concentration of lysine, alanine, glycine, serine, tyrosine, threonine, glutamate, arginine, leucine, valine, glutamine and aspartate as pregnancy progressed. McEvoy-Bowe and co-workers (1987), found that amino acid concentration in rat amf was higher than in human amf. At day 19, the total free amino acid content in rat amf was approximately 2-fold higher than in human amf at approximately 16 weeks gestation. These authors also noted that there was a tendency for most amino acids in rat amf to increase from day 17 to day 20 of gestation.

Furthermore, the amf concentration of all amino acids approximately doubled in concentration between day 19 and 20 of gestation but specific amino acid results were not shown.

B. Comparison of amniotic fluid amino acids with maternal/fetal plasma amino acids

A potential diagnostic tool in assessing the growth and metabolic status of the developing fetus may lie in the comparison of amino acids in amf with other extracellular fluid amino acid pools during pregnancy. The amino acid values of maternal plasma, fetal plasma and amf have been compared by several authors (Cockburn et al, 1970; Reid et al, 1971; A'Zary et al, 1973) but, to date, no consistency between amf and maternal plasma amino acids has been observed. In early pregnancy (13-16 weeks) the concentration of most amino acids are greater in amf than in fetal or maternal plasma. At term, the concentrations of all amino acids in amf are less than in fetal or maternal plasma. Reid and co-workers (1971) compared amino acid levels in amf with those in maternal serum in early and late pregnancy and found that the free amino acid levels of amf did not reflect maternal plasma amino acid levels. In this study the authors reported a significant increase in threonine, glutamate, proline, alanine, valine, cysteine, methionine, tyrosine, phenylalanine, lysine, histidine and arginine in amf as compared to maternal plasma from 7-18 weeks gestation. At term, the reverse was found where threonine, serine, citrulline, alanine, α-aminobutyric acid,

valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, histidine and arginine were significantly lower in amf as compared to maternal plasma. Taurine, aspartate and glutamate showed no change from early to late pregnancy. These authors concluded that the free amino acid levels of amf did not reflect maternal plasma amino acid levels. Similarly, Cockburn and co-workers (1970) found significantly higher concentrations of amf taurine, threonine, proline, alanine, cysteine, tyrosine, phenylalanine and lysine as compared to maternal plasma amino acid levels at 15-20 weeks gestation. They also found little correlation between the amino acid concentrations in amf and maternal plasma, while a positive correlation was found only with threonine and cysteine. On the other hand, a significant positive correlation was found to exist between early fetal urine and amf amino acid levels, namely taurine, valine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and 3-methylhistidine. A'Zary and co-workers (1973) also reported a positive correlation between amino acid levels in amf with fetal urine and in addition a positive correlation existed between amf amino acids and fetal serum. These findings suggest that (1) amf amino acids are a more accurate mirror of fetal metabolic status than is maternal plasma and (2) amf amino acid levels may be a better guide to the condition of the fetus at various stages of pregnancy. Recently, in a diet-induced model of fetal distress, Koski and Fergusson (1992) have suggested that amf glucose may be a more accurate mirror of fetal glycogen reserves and may be a better guide to the condition of the fetus than fetal or maternal plasma glucose concentrations. Reductions of maternal dietary carbohydrate were associated with reductions in amf glucose and fetal liver

glycogen but no significant disturbances were observed to the plasma glucose concentrations in either the fetal or maternal systems. This suggests that fetal and maternal plasma glucose concentrations were not as sensitive as amf glucose concentrations to perturbations in glucose homeostasis during pregnancy.

C. Response of amniotic fluid to nutritional deprivation

The observation that the fetus swallows amf suggests that it may have an important role in fetal nutrition (Pitkin and Reynolds, 1975; Mulvihill et al, 1985). In the later part of pregnancy the volume of amf swallowed daily by the fetus is considerable (750mL/d) and may be a significant source of nutrition for normal fetal growth and development. The fetal intestinal epithelium has the capacity to absorb and utilise carbohydrate and protein from the swallowed amf (Pitkin and Reynolds, 1975; Charlton-Char and Rudolph, 1979; Lev and Orlic, 1972).

Mulvihill and co-workers (1985) showed that prevention of fetal swallowing, following oesophageal ligation in rabbit fetuses, resulted in 14% reduction in birth weight and 10% reduction in crown-rump length compared to sham operated controls. This observation led the authors to conclude that the fetus is dependent on swallowed amf for 10% to 14% of its normal caloric intake. Pitkin and Reynolds (1975) investigated the contribution that amf protein makes to the protein requirements of the fetus. These observations bring forth the importance of swallowed amf and its significant nutritional contribution to the normal development of the fetus. Using labelled methionine injected into the amniotic sac of rhesus monkeys, the authors

estimated that amf protein contributed 10% to 15% of fetal nitrogen requirements and suggested that ingested amf protein may play a physiological role in fetal metabolism.

Although amf has been shown to play a role in fetal nutrition, little is known as to whether amf composition is altered as a result of compromised maternal nutritional status. One of the first studies to address the issue of the effects that maternal caloric deprivation has on the levels of metabolic fuel in amf was conducted by Kim and Felig (1972). These authors provided evidence that 4 days of maternal starvation resulted in decreased plasma glucose and increased plasma free fatty acids, β-hydroxybutyrate and acetoacetate concentrations. Similarly these findings were reflected in changes in amf composition. Amf glucose levels were significantly reduced in the fasted subjects with increases in the concentration of β-hydroxybutyrate and acetoacetate. They did not observe an increase in amf free fatty acid level despite the marked increase in maternal plasma free fatty acids. This is in keeping with the fact that there is limited transport of free fatty acids across the placenta and changes in maternal plasma will be reflected in amf for only those substrates which readily cross the placenta.

In a second study conducted by the same group of investigators (Felig et al, 1972) showed that the levels of amino acids in amf are profoundly influenced by maternal nutrition. Maternal caloric deprivation (84-90hr fast) during the 2nd trimester of pregnancy resulted in a marked increase in the branched-chain amino acids (BCAA), valine, leucine and isoleucine and a decrease in alanine, reflecting the pattern observed in maternal plasma. The authors also noted that despite the changes in amino acid levels in amf, the relation between amf and maternal plasma amino acid concentrations

were not altered by starvation, indicating that starvation had not altered the rate of transfer of amino acids across the placenta. Similar results were observed by Tyson and co-workers (1976), who reported that the concentration of glucose and alanine in amf from women undergoing a 72hr fast decreased in a manner parallel to that of the maternal plasma. These authors did not address the consequences that these changes in amf substrate availability following nutritional deprivation might have on fetal growth and development.

Recently, Bernstein and co-workers (1992), using a maternal starvation model to induce fetal growth retardation in rats, demonstrated decreases in the glucogenic amino acids, glycine and alanine and increases in the glycine/valine ratio in amf of the growth retarded animals. Contrary to the observations made by Felig and co-workers (1972), these authors report a decrease in amf BCAA, valine, leucine and isoleucine of the growth retarded animals. The reason for this discrepancy is not clear. In addition, Bernstein and co-workers (1992) have reported that amf valine, glycine and the glycine/valine ratio predicted > 87% of fetal growth retardation.

Koski and Fergusson (1992) have shown that changes in maternal dietary carbohydrate produced changes in amf composition. More specifically, the results demonstrated that as the level of carbohydrate increased in the maternal diet there was a significant increase in amf glucose and a significant decrease in amf uric acid. The feeding of these glucose-restricted diets to pregnant rats resulted in intrauterine growth retardation. The authors found that amf glucose was positively associated with fetal weight while amf uric acid and urea nitrogen were negatively correlated with fetal

weight. These authors suggest that the composition of amf might be used as an accessible nutritional indicator of carbohydrate status in the developing fetus.

Summary

The amf pool is a highly dynamic compartment and the concentrations of amf constituents are dependent on the stage of gestation. It is suggested that the amf pool is a more accurate mirror of fetal metabolic status and may be a better guide to the condition of the fetus. This stems from the observation that little correlation was found between amf and maternal plasma constituents while positive correlations were found between amf and fetal plasma and fetal urine constituents. Although amf has been shown to play a role in fetal nutrition little is known as to whether the amf composition is altered as a result of compromised maternal nutritional status. Those studies, which have addressed this issue, found that amf constituents, particularly glucose, alanine and the BCAAs, leucine, isoleucine and valine were influenced by maternal nutritional status. These changes in amf constituents were associated with fetal growth retardation suggesting that the amf composition might be used as an indicator of fetal metabolic status during maternal nutritional deprivation.

II. PLASMA AMINO ACIDS AS INDICATORS OF NUTRITIONAL STATUS

A. Use of plasma amino acids for diagnosing nutritional disturbances

In contrast to amf amino acids, plasma free amino acids have long been recognised as biochemical markers for the assessment of nutritional status. The

detection of perturbations in steady-state levels of amino acids in plasma has been utilised as a sensitive indicator of endocrine and metabolic adaptations involved in various physiological and pathological conditions (Saunders et al, 1967; Scriver et al, 1971; Coward and Lunn, 1981). The determination of the amino acid profile in plasma has found useful applications in evaluating nutritional disturbances such as the kwashiorkor and marasmic forms of protein-energy malnutrition and prolonged starvation (Whitehead and Dean, 1964; Saunders et al, 1967; Padilla et al, 1971; Scriver et al, 1971). The rapid response of the plasma amino acid profile to nutritional disturbances has provided one with an easily accessible experimental and clinical tool, which has contributed to a better understanding and diagnosis of the type and severity of the nutritional status.

The concentration of amino acids in plasma of children with kwashiorkor and marasmus has shown pronounced abnormalities, and it is the alterations in the pattern of plasma amino acids which has been used to distinguish between kwashiorkor and marasmus (Whitehead and Dean, 1964; Whitehead, 1969). The ratio of non-essential amino acids (NEAA) to essential amino acids (EAA) rise in children with kwashiorkor, however no such elevation is seen in marasmic children as the changes in amino acid levels are less pronounced (Whitehead and Dean, 1964; Padilla et al, 1971). Children with kwashiorkor have shown depression of certain EAA, specifically the branched-chain amino acids, valine, leucine and isoleucine, as well as decreases in methionine and a decrease in the ratio of tyrosine:phenylalanine. Among the NEAA, those associated with the urea cycle, in particular ornithine and arginine, were also reduced in

patients with kwashiorkor as compared to control subjects (Padilla et al, 1971). It has also been observed that the non-essential amino acids, alanine, glycine, serine and proline remain normal or even rise, which has led to the statement that the ratio of NEAA to EAA can be used as an index of the child's nutritional condition (Whitehead and Dean, 1964; Edozien, 1966; Saunders et al, 1967; Young et al, 1990).

Several reports have questioned the diagnostic value of plasma NEAA to EAA ratio as a biochemical index to distinguish between the type and severity of protein-calorie malnutrition (Edozien, 1966; Saunders et al, 1967; McFarlane et al, 1969; Padilla et al, 1971). These inconsistencies have been attributed to the confounding effects of metabolic and/or dietary parameters. It would appear that the malnourished subjects were undergoing refeeding with a high protein diet at the time that blood samples were collected. The results therefore were not representative of fasting plasma amino acid levels of the children on the kwashiorkor-producing diets. A fasting period of considerable length is necessary if the effects of a meal containing protein are to be considered. For example, it has been found that the alanine level in plasma is correlated with the caloric and carbohydrate content of the diet (Swenseid et al, 1967). Edozien (1966) suggested that estimation of single amino acids by themselves is not diagnostic of kwashiorkor. His findings showed that the determination of the level of total plasma amino acids was a more valuable biochemical index of the status of protein nutrition. The total amino acid levels in his study were found to be decreased even in cases which still had normal total serum protein and albumin concentrations. Therefore, it has been recommended that the total plasma amino acids as well as the ratio of NEAA/EAA be

used as an adjunct to routine clinical examinations. Most importantly, plasma amino acids may serve as a measure of the efficacy of various therapeutic diets (Whitehead and Dean, 1964).

The alterations in plasma amino acids during prolonged starvation have been extensively studied and the characteristic changes that occur in the pattern of plasma amino acids differ from those of protein-energy malnutrition. In prolonged starvation the measurement of total α-amino nitrogen reveals a relatively modest decline, reaching statistical significance only after several weeks of fasting (Felig et al, 1970). However, analysis of specific amino acids indicates that individually these amino acids respond in varying magnitudes and directions. Specifically, in the initial few days of starvation there is a transient rise in the plasma concentration of the branched-chain amino acids, particularly valine, leucine and isoleucine, a rise in methionine and α-aminobutyrate, as well as a dramatic decline in alanine and a delayed increase in glycine and threonine (Adibi, 1968; Adibi and Drash, 1970; Felig et al, 1970; Scriver et al, 1971). As starvation progresses most amino acids ultimately decline. It is believed that the hyperaminoacidemia seen early in starvation can be attributed to the concurrent fall in serum insulin, characteristic of starvation. The latter changes reflect an increased peripheral release of threonine and glycine and increased splanchnic utilisation of alanine via gluconeogenesis (Felig et al, 1970; Scriver et al, 1971).

B. Assessing pregnancy outcome using free amino acids in maternal and fetal plasma

The influence of pregnancy on the concentration of free amino acids in maternal plasma at various stages of pregnancy has been reported. Free amino acid concentrations have been observed to decrease in the maternal plasma during gestation (Reid et al, 1971). It was originally thought that the resulting hypoaminoacodemia was due to the ever-increasing demands for substrates of the rapidly growing fetus (Young and Prenton, 1969). More recently, it has been shown that this decrease in maternal plasma amino acids is already well established as early as the first trimester in women (Schoengold et al, 1978) or the 12th day of pregnancy in rats (Palou et al, 1977; Pastor-Anglada and Remesar, 1986). This is the period in which the metabolic weight of the feto-placental unit is negligible. In the study by Schoengold and co-workers (1978), of the 24 plasma amino acids measured in women, half of these amino acids were significantly lower by the end of the first trimester. These amino acids included hydroxyproline, threonine, serine, glutamate, proline, citrulline, glycine, alanine, cysteine, ornithine, tyrosine, and arginine (see Table 1). By the 12th day of pregnancy in rats, alanine, glutamine+glutamate, hydroxyproline, proline and cysteine decreased significantly and then showed slight increases before parturition, with the exception of proline and cysteine which continued to decrease with advancing gestational age (Palou et al, 1977). With most other amino acids there was a decreasing trend in concentration which was maintained throughout pregnancy, with tryptophan, taurine, valine, phenylalanine

and lysine reaching statistical significance at day 21 of pregnancy in rats (see Table 2) (Palou et al, 1977). Mechanistic explanations for the normal fall in maternal plasma amino acid concentrations has been attributed to changes in circulating hormones (Dancis et al, 1968), resulting in an increased capacity of amino acid extraction by maternal liver (Pastor-Anglada et al, 1986; Pastor-Anglada et al, 1987) or to an increased rate of placental transfer of amino acids (Carter et al, 1991).

Table 1. Plasma amino acid concentrations in women throughout pregnancy and in non-pregnant control subjects[1,2].

Amino acids	Non-pregnant	1st Trimester	2nd Trimester	3rd Trimester
		µmol/100mL		
tryptophan	6.69	5.31	6.05	5.19
arginine	9.00	6.10 ↓	5.29	4.36
taurine	9.93	6.96	5.06	4.17
hydroxyproline	1.53	1.09 ↓	1.11	1.16
aspartic acid	0.58	0.72	0.65	0.65
asparagine	5.82	4.48	5.49	5.22
threonine	14.67	11.95 ↓	14.83	18.15
serine	11.36	8.57 ↓	7.28	6.95
glutamine	53.29	42.03 ↓	37.21	39.22
glutamic acid	4.41	3.82	4.11	3.82
proline	19.68	13.61 ↓	12.88	12.04
citrulline	3.57	2.06 ↓	2.17	1.84
glycine	25.44	15.95 ↓	13.72	12.92
alanine	38.38	30.03 ↓	32.58	35.28
valine	20.05	17.44	14.85	13.72
cysteine	4.48	3.40 ↓	3.95	2.83
methionine	2.38	2.25	2.13	1.98
ornithine	5.45	3.79 ↓	2.87	2.73
isoleucine	5.71	5.28	4.59	4.32
leucine	11.40	10.64	8.42	7.51
tyrosine	6.78	5.51 ↓	4.48	4.11
phenylalanine	5.66	5.58	4.94	4.56
lysine	19.06	19.08	17.05	16.30
histidine	8.13	8.26	8.30	8.47

[1] (Schoengold et al, 1978)

[2] (↓ indicates significant decrease vs. controls)

Table 2. Plasma amino acid concentrations in day 12 and day 21 pregnant rats and non-pregnant controls[1,2,3].

Amino acids	Control	Day 12	Day 21
		µmol/L	
tryptophan	256	210	137 ↓
arginine	221	179	162
taurine	479	389	271 ↓
hydroxyproline	54	32 ↓	43
asp + asn	187	156	220
threonine	230	200	220
serine	387	358	392
glu + gln	977	838 ↓	945
proline	362	212 ↓	203 ↓
citrulline	85	96	81
glycine	272	230	221
alanine	778	501 ↓	892
valine	202	187	151 ↓
cysteine	107	90 ↓	85 ↓
met + orn	127	165	141
ile + leu	312	359	386 ↑
tyrosine	146	151	130
phenylalanine	62	54	45 ↓
lysine	515	467	349 ↓
histidine	113	82	85

[1] (Palou et al, 1977)

[2] (↓ indicates significant decrease vs. controls)

[3] (↑ indicates significant increase vs. controls)

Normal fetuses show plasma amino acid concentrations significantly higher than those of their mothers (Palou et al, 1977; McIntosh et al, 1984; Kamoun et al, 1985; Cetin et al, 1990). McIntosh and co-workers (1984) found that all essential and nonessential amino acid concentrations were consistently higher in human fetal plasma than in maternal plasma. Their results showed that the ratio of fetal to maternal amino acid concentrations varied greatly from 1.1:1 for glutamic acid to 3:1 for lysine. In 21 day fetal rats, the concentrations of individual amino acids were considerably higher than in their mothers (Palou et al, 1977). The high plasma fetal/maternal amino acid concentration ratios were seen especially with lysine, phenylalanine and hydroxyproline, suggesting a decreased amino acid oxidation (Palou et al, 1977). The high fetal to maternal amino acid concentration ratio is consistent with the presence of active amino acid transport systems within the placenta. The transport from mother to fetus occurs against a concentration gradient and involves energy-dependent transport mechanisms (Young and Prenton, 1969; Cetin et al, 1990; Hay, 1992). Thus, amino acids cross the placenta and are concentrated on the fetal side, giving rise to the high fetal/maternal amino acid concentration ratios.

The detection of abnormal changes in the amino acid levels has been useful in assessing pregnancy outcome. In this section the potential significance of maternal nutritional status on the profile of amino acids in the maternal and fetal plasma will be reviewed. This will provide a better understanding of the maternal and fetal metabolic adaptations which occur as a result of altered nutritional conditions.

C. Nutritional influences on amino acid profiles in maternal and fetal plasma

The maintenance of fetal glucose homeostasis is dependent on maternally supplied glucose. When maternal nutritional status is compromised, resulting in decreased substrate availability, the fetus becomes less reliant on transplacentally acquired glucose as a source of energy. However, because fetal glucose utilisation rates remain high and oxygen consumption does not decrease in the face of inadequate substrate availability, the fetus must rely on alternative fuel sources to maintain normoglycemia (Bassett, 1980; Lemons and Schreiner, 1984; Hay et al, 1984; Liechty et al, 1987). In the fed state, the oxidation of amino acids for fuel is a normal process of intrauterine fetal growth (Battaglia and Meschia, 1986; Cetin et al, 1992). Fetal protein synthesis calculations have shown that the uptake of amino acids by the fetus exceeds the rate needed for fetal protein accretion. This finding, together with the observation of a high rate of fetal urea production suggests that amino acids are oxidised for fuel during normal fetal development (Cetin et al, 1992). Although amino acids contribute a relatively small portion of potential oxidative substrates in the fed state, approximately 25% of fetal oxygen consumption (Lemons et al, 1976), amino acids may comprise a significantly greater source of energy substrate to the fetus during nutritional deprivation. Liechty and co-workers (1987) calculated that when exogenous glucose supply to the ovine fetus is curtailed, the reliance on amino acids as alternate sources of energy can account for as much as 55% of fetal oxygen consumption. This represents a dramatic augmentation in amino acid catabolism which may be oxidised directly to

maintain fetal energy balance or metabolised via gluconeogenesis for maintenance of glucose homeostasis (Hay et al, 1984; Kaloyianni and Freedland, 1990; Hay, 1991).

The changes in the maternal plasma amino acid profile that occurs as a result of nutritional deprivation have been documented in several different species (Lemons and Schreiner, 1984; Liechty and Lemons, 1984; Johnson et al, 1986; Domenech et al, 1986). The general finding is that the changes in the maternal plasma aminogram are confined to specific amino acids, principally the branched-chain amino acids (BCAA), leucine, isoleucine and valine and the gluconeogenic amino acid, alanine. The concentration of most amino acids decline in the maternal plasma, the most significant depletions are seen with the principal gluconeogenic amino acids, alanine and serine. In contrast, significant increases in the BCAA (leucine, isoleucine, valine) as well as glycine, lysine, phenylalanine, tyrosine, ornithine, methionine, histidine, 3-methlyhistidine and taurine have been observed in the rat (Girard et al, 1977; Arola et al, 1982; Johnson et al, 1986), sheep (Lemons and Schreiner, 1984; Liechty and Lemons, 1984) and in human gestation (Felig et al, 1972; Tyson et al, 1976). Alterations in maternal plasma amino acid concentrations during starvation are more marked in pregnant than nonpregnant subjects. Felig and co-workers (1972) observed that pregnancy accelerates and exaggerates the hypoalaninemic response to starvation. This led the authors to suggest that lack of this substrate contributes to the fasting hypoglycaemia. Furthermore, the lack of endogenous circulating alanine may be responsible for the failure of hepatic gluconeogenesis to increase adequately to maintain euglycaemia in the face of combined maternal and fetal requirements.

The changes in the concentration of amino acids within the fetal plasma in response to maternal fasting are quite different from those observed within the mother. In two separate studies, increases in the concentration of total plasma amino acids were observed in the ovine fetuses after 5 days of maternal fasting (Lemons and Schreiner, 1983) and in term fetal rats after 24 hours of maternal fasting (Girard et al, 1977). This would suggest that the fetus seems to be able to regulate the pattern of its circulating amino acid supply. This fetal metabolic adaptation to maternal fasting can be partially explained by the observation that exogenous uptake of free amino acids by the fetus from the mother does not change (Lemons and Schreiner, 1983). Recently, the observations made by Bernardini and coworkers (1991) contradict this finding. These authors report that the concentrating process of amino acids in the fetal circulation requires energy and causes of impaired energy production, as in starvation, reduces the capacity of the fetus to obtain amino acids. However, the fact that fetal urea nitrogen excretion increases and fetal oxygen consumption remains constant indicates that the fetus must catabolize a major portion of the amino acids supplied from the mother, which were previously intended for protein synthesis within the fetal compartment or alternatively the fetus must be catabolizing its own protein stores, a process which compromises fetal growth (Lemons and Schreiner, 1983; Liechty and Lemons, 1984).

Lemons and Schreiner (1983) observed that after 5 days of maternal fasting, the glucogenic precursor alanine, which was found to be lower in the maternal circulation, was higher in ovine fetal plasma; however this effect was not found to be statistically significant. These authors reported a significant increase in the BCAA, leucine,

isoleucine and valine, as well as increases in taurine, methionine, histidine, phenylalanine, lysine and 3-methylhistidine. The increase in fetal plasma concentration of 3-methylhistidine suggested an augmentation of protein breakdown within fetal muscle tissue. The only significant decreases observed were that of glutamate and arginine. In the study by Girard and co-workers (1977), pregnant rats were fasted for 24, 48 or 96 hours and fetal plasma samples were obtained at day 21 of gestation. The results showed a significant decrease in alanine and serine levels in fetal plasma during maternal fasting and a rise of the BCAA, leucine, isoleucine and valine, a rise in glutamine, lysine, taurine and glycine also occurred. Contrasting results were found in the work by Johnson and co-workers (1986), who studied the effects of maternal fasting (96 hours) upon fetal plasma amino acids in rats at term (day 21). The authors reported a significant decrease in total fetal plasma amino acids, a significant decrease in alanine, glutamate, citrulline and valine and a significant decrease in the sum of BCAA (leucine, isoleucine and valine). It was suggested that these differences were due to the length of the fast and the stage of gestation studied.

The above changes detail the adaptations of near-term fetuses. However, limited information is available for earlier time points during pregnancy, when questions about the prognosis of fetal outcome might be raised. The study by Arola and co-workers (1982) addressed the issue of the effect of 24 hours of starvation upon fetal plasma amino acid composition at two critical time points, day 19 and 21 of gestation in rats. The results showed that the combined essential amino acids decreased by 14% in 19 day fetuses and actually increased in 21 day fetuses. While all the gluconeogenic

amino acids decreased significantly, the degree of drop was greater in 21 day fetuses vs. 19 day fetuses. It appeared that day 19 fetuses were well protected by the maternal organism during starvation probably due to their lower needs compared to day 21 fetuses. The authors concluded that 21 day fetuses have a greater ability to concentrate amino acids than day 19 fetuses, that is, they are better at keeping their own homeostasis of nutrients than day 19 fetuses. However, day 21 fetuses were affected by marked growth retardation.

III. IUGR AND PREDICTORS OF FETAL GROWTH

A. Predicting fetal growth

Intrauterine growth retardation (IUGR), also termed intrauterine malnutrition, is one of the most important problems in obstetric practice. Small for gestational age (SGA) infants have an increased incidence of perinatal morbidity and mortality as well as ongoing childhood illnesses (van den Berg and Yerushalmy, 1966; Villar, 1982). There have been ongoing efforts to develop biochemical tests that could be used to assess fetal growth and organ maturity, such as assessing fetal lung maturity. The analysis of maternal and fetal plasma and amf may aide in deciding the safest time to deliver those infants of high risk pregnancies. Therefore, it is of importance that there be clinical and technical methods available for early screening and assessment of IUGR.

Maternal plasma amino acids have been used as metabolic markers for the assessment of fetal growth and maturity. Moghissi and co-workers (1975), identified that the concentration of certain maternal plasma amino acids in the 3rd trimester of pregnancy correlated significantly with fetal growth and development. Among the amino acids, the authors showed that glycine, lysine and total plasma amino acids were positively correlated with birth weight, whereas valine and threonine showed a negative correlation. Cranial volume at birth was significantly and positively correlated with glycine and glutamine and negatively correlated with threonine, histidine and glutamate. The authors suggested that the concentration of amino acids in maternal plasma, particularly glycine, lysine and histidine in late pregnancy may be valid predictors of fetal growth and development. The work of Crosby and co-workers (1977) on an appraisal of correlated factors affecting fetal growth found that among a group of 182 pregnant women studied at midpregnancy, maternal plasma concentrations of aspartic acid, phenylalanine, lysine, histidine and arginine were significantly and positively correlated with adjusted birth weight and the relationship between maternal plasma amino acids and fetal birth weight was seen as early as the 1st trimester of pregnancy. McClain and co-workers (1978) observed significant positive correlations between maternal plasma levels of aspartic acid, serine, alanine, tyrosine, ornithine and arginine and a negative correlation with methionine, valine and isoleucine and fetal size at birth. For those mothers who subsequently gave birth to infants with low weight/gestational age ratios and clinical evidence of fetal malnutrition (FM), these authors reported significantly lower levels of 10 of the 18

maternal plasma amino acids measured as compared to mothers giving birth to normal babies. The amino acids affected were aspartate, serine, glutamate, alanine, tyrosine, phenylalanine, ornithine, lysine, histidine and arginine. Not all of these amino acids were reduced in the same proportion. Ornithine, arginine and aspartate were over 30% lower in FM mothers than the normal mothers. The low ornithine and glutamate levels present in plasma from FM mothers were suggested to reflect an active urea cycle. The significantly lower concentration of aspartate, glutamate and alanine likely indicated elevated gluconeogenesis and thus the necessity for an active urea cycle. In addition, the total amino acid concentrations were found to be almost 20% lower in the FM mothers. These observations support the use of maternal plasma amino acids for the early detection of fetal growth retardation.

With the advent of techniques for sampling fetal plasma at midgestation, either during fetoscopy or by cordocentesis, it has become possible to measure fetal plasma amino acid concentrations. Cetin and co-workers (1990) observed significant alterations in the concentrations of certain amino acids in the plasma of small-for-date fetuses (SGA) as early as the 27th week of gestation. Specifically, a significant reduction in α-aminonitrogen was found in SGA fetuses sampled in utero as compared with normal fetuses. In addition, these authors observed that the neutral branched chain amino acids (BCAA), leucine, isoleucine and valine were reduced in SGA fetuses. These authors suggested that the BCAA were extensively used within the fetus and placenta for both protein synthesis and energy production. In this same study, it was

also shown that phenylalanine, histidine, arginine and alanine concentrations were significantly higher in the plasma of mothers of SGA infants.

B. Diagnosing fetal growth and metabolic status using amniotic fluid

The increasing availability of performing amniocentesis at various gestational times has made the analysis of amf constituents of value in the clinical investigation of fetal growth and metabolic status. Biochemical analysis of the fetal fluid environment may reflect physiological and pathological fetal states. Glucose and amino acid homeostasis in amf is essential for normal fetal growth and development. Abnormal glucose and amino acid concentrations or amino acid ratios indicate abnormal fetal development, which may be caused by a number of factors; such as genetic defects, teratogenic agents and the nutritional state of the mother (Mesavage et al, 1985).

The examination of amf, especially early in pregnancy, may provide early warning of pending fetal problems or abnormal fetal development. Fetal growth retardation (IUGR) is a complication of pregnancy that is associated with increased perinatal morbidity and mortality. In the assessment of IUGR, it has been observed that lowered amf glucose, glucose concentrations below 5mg/dL, was associated with fetal growth retardation (Drazancic and Kuvacic, 1974). Wharton and co-workers (1971) reported that total amf hydroxyproline concentration tended to be lower in those pregnancies producing small-for-date babies and those at particular risk of IUGR. Recently, in a diet-induced model of fetal distress, it was shown that higher amf glucose concentrations were associated with increased fetal weight, whereas higher

amf uric acid concentrations were correlated with lower fetal weight (Koski and Fergusson, 1992).

Specific amf constituents have been used in the assessment of fetal maturity, and fetal defects, such as central nervous system (CNS) malformations. It is suggested that renal maturity, as reflected in the creatinine and uric acid concentrations in amf, may indirectly provide an assessment of fetal maturity. This stems from the fact that creatinine and uric acid increase in amf with gestational age, i.e., with increasing maturation of the urinary system. Teoh and co-workers (1973) quantified the levels of amf creatinine and uric acid, which were indicative of fetal maturity. These authors reported that a creatinine concentration of 2.0mg/100mL could be used to pick up 53% of "mature" fetuses (fetuses which had passed the 37th week of gestation) and a uric acid concentration of 5mg/100mL correctly indicated a mature fetus in 42% of patients.

Fetal age, as estimated by crown-rump measurements, has been shown to be correlated with specific amf amino acids. Free amino acids in amf were quantified in human fetuses of 48-140 days gestation (Scott et al, 1972). Fourteen of the twenty-six amino acids, namely, lysine, leucine, phenylalanine, isoleucine, valine, ornithine, alanine, tyrosine, methionine, arginine, serine, histidine, proline and taurine showed statistically significant changes in concentrations which correlated inversely with fetal age. The authors further observed that the concentration of lysine showed the highest degree of correlation with fetal age, with a correlation coefficient of 0.89. In a recent study, Mesavage and co-workers (1985) examined amf amino acids in normal pregnancy between the 13^{th} and 23^{rd} week of gestation. A negative correlation with

gestational age was found for leucine, valine, isoleucine, phenylalanine, lysine, alanine, aspartate, tyrosine, glutamate and proline, with leucine showing the greatest rate of change.

IV. CONCLUSION

Review of the literature revealed that maternal nutritional status has an impact on the profile of certain amf and maternal/fetal plasma constituents during pregnancy. Glucose, alanine and the BCAAs, leucine, isoleucine and valine are those amf and plasma constituents which are most commonly analyzed and been shown to be altered by maternal nutritional status. There is a lack of information regarding a more complete profile of amf constituents, specifically amf amino acids, particularly during compromised maternal nutritional status. Changes in the composition of amf and plasma constituents have been shown to provide a useful diagnostic tool in monitoring fetal growth and metabolic status. The amf pool has been suggested to be a more accurate mirror of fetal metabolic status. Therefore there is clearly a need for more research in determining the effect of compromised maternal nutritional status on amniotic fluid constituents and whether these changes can be used to predict fetal development.

V. STATEMENT OF PURPOSE

The amniotic fluid (amf) pool represents a nutritional reservoir for the developing fetus and contributes in part to fetal growth (Mulvihill et al, 1985). Little is known as to whether compromised maternal nutritional status alters this compartment and whether any resulting changes in the profile of amf constituents are predictive of fetal development. Those studies which have examined the effects of maternal fasting on fetal development, have found that maternal fasting resulted in impaired fetal growth and reduced fetal heart (Shelley, 1961) and lung (Rhoaded and Ryder, 1981) glycogen content. No information is available which examines the relationship between maternal dietary glucose availability, glucose being an essential nutrient during pregnancy (Koski and Hill, 1990), amf amino acid composition, and fetal growth and metabolic status. Previous studies from our laboratory have shown that the feeding of glucose-restricted diets to pregnant rat dams resulted in fetal growth retardation (Koski and Fergusson, 1992; Lanoue, 1993). Fetal growth retardation was accompanied by changes in amf glucose concentrations and amf glucose was found to be predictive of fetal growth parameters (body weight, lung weight and heart weight) and fetal tissue glycogen content (liver, heart and lung). It is not known whether this same dietary model produces changes in the concentrations of amf amino acids and whether amf amino acids can be used to predict the impact of glucose restriction on fetal growth. The purpose of this study was to investigate the effects of maternal dietary glucose

restriction on amf composition, specifically the composition of amf amino acids in rats. In addition, this study investigated whether any alterations in amf amino acids could be used to monitor fetal growth and metabolic status. The hypothesis was that limited maternal dietary glucose would result in altered amf amino acid composition, which in turn could be predictive of fetal growth and metabolic status. Historically, maternal caloric deprevation has resulted in alterations of specific amf amino acids, namely alanine, glycine and the branched-chain amino acids valine, leucine and isoleucine (Felig et al, 1972; Bernstein et al, 1992). The amino acids taurine and histidine are often those amino acids used to explain the metabolic adaptations that occur to maintain glucose homeostasis in response to maternal starvation (Meschia, 1978; Johnson et al, 1986). The specific objectives of this study were therefore designed to answer the following questions:

1) Does limited maternal dietary glucose result in decreased amf glucogenic amino acids, principally, alanine and glycine, increased amf branched-chain amino acids, valine, leucine and isoleucine and decreased amf taurine levels?

2) Do amf taurine and histidine correlate with fetal growth?

3) Can amf constituents predict fetal growth and metabolic status?

CHAPTER 2 - MATERIALS AND METHODS

A. EXPERIMENTAL DESIGN

In this experiment, graded levels of dietary carbohydrate (0%, 12%, 24% and 60%) were fed to pregnant rats throughout pregnancy and their effects on amniotic fluid amino acids were studied during the last four days of gestation (days 18.5, 19.5, 20.5 and 21.5). Impregnated Sprague-Dawley rats (Charles River Canada, St-Constant, Quebec) weighing 180-220g were received within two days of mating (gestational day 0 was defined as the day following the overnight mating), and randomly assigned to one of the sixteen experimental groups.

B. EXPERIMENTAL DIETS

Dams were randomly assigned to one of four experimental diets (0%, 12%, 24% and 60% glucose). The carbohydrate-restricted diets were formulated by isocaloric substitution of dietary glucose with triglyceride from soybean oil as previously described by Koski and coworkers (1986a,b). Cellulose was added to maintain diets isocaloric which supplied 17.4kJ of metabolizable energy per gram dry matter as recommended for pregnant rats (NRC 1978). The amount of casein added to the diets was calculated to provide 11% of protein, which is the lowest level required to supply the requirement of essential amino acids during pregnancy for

optimal fetal growth and to prevent any excess protein from being transformed to glucose via gluconeogenesis (Koski et al, 1986). Approximately four times the NRC requirements for the water-soluble vitamins and 1.5-2 times the NRC requirements for the fat-soluble vitamins were added to each diet. This was to ensure that ample vitamins were supplied to those dams in the glucose free and glucose restricted group since food intake might be lower in these dietary groups. Since the dietary requirement for choline is influenced by the lipid content of the diet, it has been suggested that the choline content of high fat diets be increased (NRC 1978). For this reason 0.4% choline was added instead of 0.1% provided by normal adequate diets. The salt mix was added at a dietary level of 5.5%. Each essential mineral was provided at a dietary level twice that estimated as the requirement for gestation by NRC (1978). Some modifications of salts added were made based on previous reports. Manganese and zinc values corresponded to values reported by Keen and coworkers (1983) to have historically demonstrated reproductive performance. The copper value was chosen at 9ppm since values below this level have been shown to be inadequate for maintaining optimal copper levels in the weanling pup (Cerklewski, 1979). Supplements of sodium bicarbonate were added at 1% dietary level to correct for any metabolic acidosis that might occur from feeding a carbohydrate-free and carbohydrate restricted diet.

The rational for the formulation of the diets selected was based on work by Koski and coworkers (1986), who demonstrated that a 0% glucose diet was associated with high postnatal mortality; 12% was the minimal level of dietary

glucose that produced normal fetal weight at term; 24% glucose diet was investigated since it was previously shown that fetuses from dams fed 12% glucose accumulated significantly less liver glycogen than fetuses from control dams (Lanoue, 1993), suggesting that this level of carbohydrate was not adequate when fetal liver glycogen concentration was used to evaluate dietary adequacy. The 60% carbohydrate diet served as the standard control used in all reproductive studies.

C. COLLECTION OF AMNIOTIC FLUID

The dams were anaesthetized with ketamine-HCl (Rogarsetic, 30mg/kg, Rogar/STB, London, Ont.) into the jugular region to avoid anaesthetizing the fetuses. A cesarean section was performed on all dams and amniotic fluid was collected with tuberculin syringes from the amniotic sac of several conceptus of each litter and pooled to yield approximately 1mL sample. Amniotic fluid was stored at -80°C until analyzed.

D. ANALYTICAL PROCEDURES

i) Sample Deproteinization and Preparation

The method described below for the deproteinization of amniotic fluid samples is one that is a composite of the methods described by Hubbard and coworkers (1988) and Teik Ng (1991). Briefly, amniotic fluid samples (500µL) were

placed individually in 1.5mL Eppendorf tubes and deproteinized with 5-sulfosalicyclic acid (SSA) solution (35% w/v). The tubes were capped, vortexed for 5 seconds, allowed to stand at room temperature for 5 minutes and then centrifuged at 15000g for 15 minutes (Microspin 12S Sorvall). The protein-free supernatants were filtered through a 0.2μm Z-Spin microcentrifuge filter reservoir (Gelman Sciences, #S4395), and then were centrifuged at 3860g for 15 minutes at 4°C (Sorvall RT6000B). This deproteinization method alters the pH of the sample solution which has considerable effect on the elution times and peak fraction volumes of some acidic amino acids, particularly when lithium buffers are used for eluting the resin column (Mondino et al, 1972). For this reason, the ultrafiltrates were evaporated to dryness, using a rotary evaporator set at 25°C for approximately 2 hours or until samples were completely dry. The residue was then dissolved in 1mL Li-S buffer (pH=2.2) to which 166.67μL of Nor-Leucine™ (700nmol/mL) was added. Nor-Leucine™ served as the internal standard for the amount of sample injected into the amino acid analyzer. A volume of 350μL of the sample (having a final pH=2.2) was drawn into a loading loop of the Beckman System Model 121M Amino Acid Analyzer.

ii) Amino Acid Standard Preparation

A standard mixture, STD Hydrolyzate Standard (Beckman), containing 18 components; an AN^+, Acidic and Neutral Amino Acid Supplement (Beckman)

containing 13 acidic and neutral amino acids; and a B^+, Basic Amino Acid Supplement (Beckman) containing 9 basic amino acid components were used for the preparation of a calibration standard solution. Each of the amino acids was present at a concentration of 2500nmol/mL except where otherwise indicated. Each of the STD, AN^+ and B^+ standards were diluted with lithium (Li-S) diluting buffer (pH2.2) to a final concentration of 700nmol/mL. Glutamine, asparagine and tryptophan were not included in any of the calibration standards above since these amino acids are unstable at low pH and therefore are not included in these acidic solutions. Separate solutions of each were prepared, having a concentration of 700nmol/mL each. Nor-Leucine™ (700nmol/mL) was used as the internal standard. An equal volume of the standards (STD, AN^+, and B^+), the individual amino acids (glutamine, asparagine and tryptophan) and Nor-Leucine™ were combined and served as the standard solution (100nmol/mL). This amino acid standard solution was used for calibration during quantitative analysis.

E. AMINO ACID ANALYSIS

i) Ion-Exchange Chromatography

Ion-exchange chromatography with ninhydrin detection was used for the separation and quantitative determination of the mixture of amino acids present in amniotic fluid. The separation of amniotic fluid amino acids was effected by the use

of a sulfonated cation exchange resin. Depending on their dissociation characteristics, the amino acids were differentially eluted from the ion-exchange resin column using a series of lithium buffers of increasing pH and ionic strength. To provide optimal resolution of all the amino acids, a sequence of two column temperatures was employed. In the early part of the analysis a low temperature (38.5°C) was used for the resolution of the acidic and neutral amino acids. A switch to a higher temperature (64.5°C) in the latter part of a run was used for the remaining amino acids (Slocum and Cummings, 1991). Visualization of the individual amino acids was accomplished via post-column ninhydrin derivatization and colorimetric detection. Simultaneous dual-channel detection was provided to allow for quantitation of the secondary amino acids namely, proline and hydroxyproline (wavelength=440nm) and the primary amino acids (wavelength=540nm) (Pfeifer et al, 1983). Amniotic fluid amino acids were tentatively identified by agreement in elution times between the peaks of amniotic fluid amino acids and those of a known reference standard mixture (Saifer et al, 1970).

ii) Operating Parameters

The following briefly outlines the technical details of the operating parameters used with the Beckman Model 121M microcolumn automatic amino acid analyzer. Refinements to the procedure described by Beckman Instruments (1975) were made in order to achieve optimal resolution of the amino acids. A single

microbore column (560x2.8mm) was packed with Beckman microcolumn cation exchange resin. The resin bed height was adjusted to 27.5cm. The column temperature was set at low (38.5°C) for the first 162.3minutes of the run and then switched to high at 64.5°C for the remainder of the run. The low column temperature of 38.5 was determined by the resolution of threonine and serine. A sequence of three lithium buffers, Li-A, Li-B and Li-C (Beckman High Performance Buffers) were used. Buffers Li-A and Li-C were used directly as provided by Beckman Specialties. The pH of Li-B buffer (initial pH=3.627) was adjusted to a final pH of 3.878 by adding approximately 3.0cc of HCl. Buffer flow rate was set at 9.4mL/hr. Ninhydrine reagent (Beckman Nin-R High Performance Ninhydrine Reagent) was prepared by stirring pre-measured vials of hydrindantin into the dimethyl sulphoxide (DMSO) ninhydrine reagent just before use. The flow rate of ninhydrin was set at 4.9mL/hr. Lithium hydroxide (0.3M) was used to regenerate the column. Amino acids were detected at two wavelengths, 440nm and 540nm. Samples were held in individual coils of small-bore tubing, which minimized the hazards of evaporation, oxidation and contamination. Samples were automatically injected at a point between the buffer solution and the resin column and became part of the eluent stream. Prepurified nitrogen was used in the Automatic Sample Injector to purge the injection valves and sample coils after they had been washed.

F. STATISTICAL ANALYSIS

The experimental design was such that a two-way analysis of variance was used to test the effects of dietary glucose levels (0%. 12%, 24% and 60%) and the effects of gestational days (18.5, 19.5, 20.5 and 21.5) and day*diet interaction in a 4*4 factorial design. Data management was carried out using Excel v. 5.0 (1993) and IBM Personal Editor v.2. All analysis was carried out using the SAS System for Windows v.6.10 (1994, Cary, NC).

Prior to any analysis of variance, amniotic fluid data was tested for homogeneity of variance using the Bartlett test (Steel and Torrie, 1980). Statistical analysis was done on transformed data for those variables that tested significant. More specifically, data with non-homogeneous variances were log transformed (tryptophan) or when this transformation did not result in homogeneous variances, observations were weighted by the reciprocal of the group variance for each of the following amino acids: ornithine, lysine and histidine. Amniotic fluid data was tested against the effect of day, diet and day*diet interaction. Differences between group means were given by least square means, probability of $p<0.05$ was accepted as significant.

Correlation analysis was carried out on fetal growth parameters (fetal weight, lung weight and heart weight) and fetal tissue glycogen reserves (fetal liver, lung and heart glycogen) and amniotic fluid amino acids. Multiple regression was done to

determine those amniotic fluid amino acids (independent variables) which may be predictors of fetal growth parameters and fetal tissue glycogen reserves as the dependent variables.

CHAPTER 3 - RESULTS

Please note that all result tables and figures can be found at the end of chapter 3 on pages 52 to 67.

A. AMNIOTIC FLUID AMINO ACID ANALYSIS - ANALYSIS OF MAIN EFFECTS

Statistical analysis of main effects of diet, day and diet*day interactions on amniotic fluid (amf) amino acid are shown in Table 3. Significant diet and day effects on amf amino acid levels were found. Significant interactions were found between day of gestation and diet indicating that the effect of day was not the same within each dietary group.

Of the 26 amf amino acid analyzed, amf isoleucine, tryptophan, 3-methylhistidine and taurine significantly responded to diet treatment. A greater number of amf amino acids significantly responded to the main effect of gestational day: cysteine, histidine, lysine, isoleucine, phenylalanine, tryptophan, tyrosine, ethanolamine, 3-methylhistidine, ornithine, phosphoserine and taurine. This suggests that amf amino acids are highly dependent on the gestational period studied. Significant interactions of diet and day were found with amf histidine, leucine, phenylalanine, tyrosine, ornithine and taurine.

Glucogenic amino acids

A significant main effect of gestational day was found with amf cysteine concentrations (see Figure 4a). Amf cysteine levels increased significantly as pregnancy progressed in those dams fed the 0% and 24% glucose diets. In dams fed the 0% glucose diet, the concentration of amf cysteine was significantly higher at term than on day 18.5 of gestation. Similarly, in dams fed the 24% glucose diet, amf cysteine concentrations progressively increased with advancing gestational days. Specifically, amf cysteine concentrations were significantly higher on both days 20.5 and 21.5 of gestation as compared to day 18.5 of gestation. No significant change occurred in dams fed the 12% and 60% glucose diets during the last four days of gestation.

A significant interaction of diet and day was found for amf histidine concentrations (Figure 5a). On day 18.5 of gestation, the difference between amf histidine concentrations of dams fed the 0% and 12% glucose diets was significantly less than the difference between amf histidine concentrations of dams fed the 0% and 12% glucose diets on day 21.5 of gestation. Although a statistical significance was found, no clear biological pattern could be described for the effect of dietary treatment on amf histidine levels during the last four days of gestation.

Ketogenic amino acids

A significant interaction of diet and day was found for amf leucine concentrations. On day 18, the difference between amf leucine concentrations of dams fed 0 and 12% dietary glucose and dams fed 12 and 24% dietary glucose was

significantly less than the difference between amf leucine concentrations of dams fed these same diets on day 19 of gestation. In addition, the differences between dietary groups (12% vs. 24% and 12% vs. 60%) were greater on day 21.5 than on day 18.5 of gestation. No clear biological pattern could be explained with the present data.

Figure 4b shows that amf lysine responded significantly to day of gestation. Amf lysine concentrations in dams fed 12% glucose were significantly higher on day 18.5 as compared to day 20.5 of gestation. In dams fed 24% glucose diet, amf lysine was significantly lower on day 19.5 as compared to day 21.5 of gestation.

Ketogenic and glucogenic amino acids

As shown in Figure 1a, dietary treatment significantly altered amf isoleucine concentrations only on day 21.5 of gestation. Complete glucose restriction (0%) resulted in significantly higher amf isoleucine concentrations than in dams fed the 12% glucose diet. No significant differences were observed between the 12%, 24% and 60% glucose diets. A significant difference was seen between days of development. Figure 1b shows that in dams fed 0% glucose diet, the concentration amf isoleucine was significantly lower on day 18.5 than on day 21.5 of gestation. With dams fed the 12% glucose diet, amf isoleucine concentrations were significantly lower on day 19.5 as compared to day 20.5, however the concentrations amf isoleucine on day 20.5 was significantly higher than on day 21.5 of gestation.

A significant interaction between diet and day of gestation was found for both amf phenylalanine and tyrosine concentrations. For both amf phenylalanine and tyrosine, the difference between all dietary groups was less on gestational day 18.5

than on days 19.5, 20.5 and 21.5. With the present data, we were not able to describe any biological pattern for the effect of dietary treatment during the last four days of gestation for both these amf amino acids.

The concentration of amf tryptophan was altered by maternal dietary treatment (see Figure 2a). On day 18.5 of gestation, amf tryptophan concentrations decreased with increasing maternal dietary glucose. In dams fed the 0% glucose diet amf tryptophan was significantly higher than in dams fed the 24% and 60% glucose diets. At term (d21.5), dams fed the 0% and 24% carbohydrate restricted diets produced significantly higher concentrations of amf tryptophan as compared to dams fed the control diet (60%). A significant effect of amf tryptophan concentrations to day of gestation was observed in dams fed the 24% glucose diets (see Figure 2b). Amf tryptophan concentrations increased with the progression of pregnancy. Amf tryptophan concentrations were significantly higher in both days 20.5 and 21.5 of gestation as compared to day 18.5.

Other amniotic fluid constituents

A significant main effect of gestational day was found with amf ethanolamine (see Figure 4c). Amf ethanolamine concentrations progressively increased with advancing gestational days in dams fed the 0% and 24% glucose diets. In dams fed the 0% glucose diet, amf ethanolamine concentrations were significantly lower on day 18.5 of gestation as compared to day 21.5. In dams fed the 24% glucose diets amf ethanolamine was significantly lower on day 18.5 as compared to days 20.5 and 21.5 of gestation.

A significant main effect of maternal dietary glucose and gestational day was found with amf 3-methylhistidine concentrations (see Figure 3a,b). On both days 20.5 and 21.5 of gestation, amf 3-methylhistidine decreased as the level of glucose increased in the maternal diet. On day 20.5, amf 3-methylhistidine concentrations were significantly higher in dams fed the 0% and 12% glucose diets as compared to the control diet (60%). At term (21.5), the concentration of amf 3-methylhistidine was significantly higher in dams fed the 0% glucose diet as compared to dams fed the 60% diet. The effect of gestational days on the concentration of amf 3-methylhistidine is shown in Figure 3b. The results show that with the carbohydrate restricted diets, 0%, 12% and 24%, amf 3-methylhistidine concentrations increased with the progression of pregnancy, no significant increase was observed with the control diet (60%). In dams fed the 0% glucose diet, the concentration of amf 3-methylhistidine was significantly higher at term (d21.5) than on days 18.5 and 19.5 of gestation. With both the 12% and 24% glucose diets amf 3-methylhistidine concentrations were significantly higher on days 20.5 and 21.5 as compared to days 18.5 and 19.5.

A significant effect of gestational day was observed for amf phosphoserine concentrations (see Figure 4d). Amf phosphoserine concentrations, in dams fed the 0% and 24% glucose diets increased with the progression of pregnancy. Specifically, in dams fed the 0% and the 24% glucose diets, amf phosphoserine concentrations were significantly lower on day 18.5 of gestation than on days 20.5 and 21.5 of

gestation. No significant effect of gestational days was observed in dams fed the 12% and 60% glucose diets.

A significant interaction between diet and day of gestation was found for amf taurine concentrations (see Figure 5b). In dams fed the carbohydrate-restricted diets, amf taurine concentrations significantly increased with the progression of pregnancy as compared to dams fed the control diet (60% glucose) throughout gestation. The differences between dietary groups were greater on gestational days 20.5 and 21.5 than on gestational days 18.5 and 19.5.

B. CORRELATION ANALYSES

The probabilities of the Pearson correlation coefficients for comparisons of amf amino acids with fetal growth parameters (fetal weight, lung weight and heart weight) are summarized in Table 8. Of the glucogenic amf amino acids, cysteine and histidine were significantly and positively correlated with all 3 fetal growth parameters, fetal weight, lung weight and heart weight. Amf methionine was significantly and positively correlated with both fetal weight and lung weight, whereas amf proline was significantly and positively correlated with only fetal heart weight.

The ketogenic/glucogenic amf amino acids phenylalanine, tryptophan and tyrosine were significantly and positively correlated with fetal weight, lung weight and heart weight.

Amf ethanolamine was significantly and positively correlated with only fetal weight. Amf 3-methylhistidine and phosphoserine were significantly and positively correlated with all three growth parameters. Amf ornithine was significantly and positively correlated with both fetal weight and heart weight. Amf taurine was significantly and positively correlated only with fetal heart weight.

The probabilities of the Pearson correlation coefficients for comparisons of amf amino acids with fetal tissue glycogen reserves (liver glycogen, lung glycogen and heart glycogen) are summarized in Table 9. The glucogenic amf amino acids arginine and asparagine were found to be significantly and positively correlated with only fetal lung glycogen. Amf cysteine was found to be significantly and positively correlated with both fetal liver and lung glycogen reserves. Amf methionine was significantly and positively correlated with liver and heart glycogen reserves. A significant and negative correlation was found with amf glutamine and fetal liver glycogen.

Amf phenylalanine and tyrosine were significantly and positively correlated with both fetal liver glycogen and lung glycogen. Tryptophan was found to be significantly and positively correlated with only fetal lung glycogen.

A significant and positive correlation was found with amf 3-methylhistidine and fetal liver glycogen, whereas amf taurine was significantly and negatively correlated with fetal heart glycogen.

C. MULTIPLE REGRESSIONS ANALYSES

The regression analyses of amf glucose and amino acids against the dependent variable of fetal weight, lung weight and heart weight are summarized in Table 10. Certain amf glucogenic amino acids were significantly associated with fetal weight and lung weight. Amf cysteine was significantly and negatively associated with fetal lung weight whereas amf methionine was significantly and positively associated with both fetal weight and lung weight. Of the ketogenic/glucogenic amino acids only amf phenylalanine was found to be significantly and positively associated with fetal lung weight. Amf 3-methylhistidine was significantly and positively associated with fetal weight only. Amf methionine and 3-methylhistidine were predictive of fetal weight and explained 59% of its variability.

The regression analyses of amf glucose and amino acids against the dependent variables of fetal tissue glycogen reserves (liver glycogen, lung glycogen and heart glycogen) are summarized in Table 11. Amf glucose was significantly and positively associated with fetal lung glycogen. Of the glucogenic amino acids, amf cysteine was significantly and negatively associated with fetal liver glycogen only whereas methionine was significantly and positively associated with both fetal liver and heart glycogen. A significant and positive association of the ketogenic/glucogenic amino acid, phenylalanine, was found only with fetal lung glycogen. Amf 3-methylhistidine was significantly and positively associated with

fetal liver glycogen whereas amf taurine was significantly and negatively associated with feat heart glycogen only. For fetal lung glycogen the model explained 39% of the variability.

Table 3. Statistical analysis of main effects on amniotic fluid amino acids

Amniotic fluid amino acids	(n)	Main Effects DIET	DAY	DIET*DAY
		F/p values	F/p values	F/p values
glucogenic				
alanine	52	1.32/0.2824	0.50/0.6847	0.84/0.5707
arginine	52	1.52/0.2255	2.65/0.0632	1.25/0.2993
asparagine	24	0.41/0.7508	0.52/0.6795	0.91/0.5225
cysteine	52	1.05/0.3815	5.20/0.0042	1.96/0.0803
glutamate	29	0.77/0.5295	1.56/0.2369	0.41/0.8627
glutamine	29	1.12/0.3686	1.08/0.3876	0.68/0.6683
glycine	52	0.24/0.8658	1.38/0.2652	2.00/0.0741
histidine	52	0.76/0.5218	30.20/0.0001	2.67/0.0295
methionine	52	1.77/0.1701	1.27/0.2983	2.00/0.0736
proline	30	0.23/0.8735	1.19/0.3434	1.27/0.3252
serine	19	0.79/0.5288	0.77/0.5360	1.09/0.3741
threonine	19	1.64/0.2424	0.79/0.5251	0.27/0.7719
valine	52	0.40/0.7571	1.61/0.2031	2.07/0.0647
ketogenic				
leucine	52	0.86/0.4684	0.15/0.9299	2.52/0.0268
lysine	52	0.21/0.8893	4.92/0.0057	2.33/0.0525
ketogenic+glucogenic				
isoleucine	52	4.47/0.0090	3.23/0.0331	1.31/0.2670
phenylalanine	52	2.78/0.0544	13.44/0.0001	2.83/0.0148
tryptophan	51	4.61/0.0079	6.97/0.0008	0.78/0.6341
tyrosine	52	1.72/0.1787	9.61/0.0001	3.84/0.0023
other				
ethanolamine	50	1.36/0.2701	3.55/0.0241	1.15/0.3575
γ-aminobutyrate	52	0.58/0.6316	0.50/0.6865	0.26/0.9763
homocysteine	52	0.87/0.4638	0.39/0.7606	1.84/0.1000
3-methylhistidine	52	7.09/0.0007	22.21/0.0001	1.95/0.0806
ornithine	52	1.85/0.1549	22.16/0.0001	3.05/0.0160
phosphoserine	51	0.50/0.6833	6.96/0.0008	0.76/0.6357
taurine	52	12.80/0.0001	8.63/0.0002	3.81/0.0024

Table 4. Amniotic fluid glucogenic amino acids (listed in alphabetical order) [1]

Dietary Glucose (%)	Days of gestation 18	19	20	21	pooled
			alanine (μ mol/L)		
0	(6) [a] 415.29±26.14[A]	(3) [a] 424.14±36.97[A]	(1) [a] 328.34±64.04[A]	(8) [a] 384.12±22.64[A]	(18) 387.97±20.41
12	(2) [ab]423.78±45.28[A]	(1) [ab]441.38±64.04[A]	(4) [a] 357.48±32.02[A]	(2) [b] 445.60±45.28[AB]	(9) 417.06±24.01
24	(7) [a] 400.20±24.20[A]	(4) [a] 392.72±32.02[A]	(3) [a] 426.26±36.97[A]	(2) [a] 452.85±45.28[AB]	(16) 418.01±17.73
60	(3) [a] 422.64±36.97[A]	---	(3) [a] 439.98±36.97[A]	(3) [a] 467.34±36.97[B]	---
pooled	(18) 415.48±17.11	---	(11) 388.02±22.16	(15) 437.48±19.33	
			arginine (μmol/L)		
0	(6) [a] 214.67±34.57[A]	(3) [a] 264.19±48.89[A]	(1) [a] 379.92±84.69[A]	(8) [a] 284.83±29.94[A]	(18) 285.90±26.99
12	(2) [ab]296.10±59.88[A]	(1) [ab] 196.56±84.69[A]	(4) [a] 332.61±42.34[A]	(2) [b] 126.17±59.88[A]	(9) 237.86±31.76
24	(7) [a] 221.31±32.01[A]	(4) [ab] 252.69±42.34[A]	(3) [b] 322.73±48.89[A]	(2) [ab] 268.94±59.88[A]	(16) 266.42±23.44
60	(3) [a] 241.37±48.89[A]	---	(3) [a] 228.79±48.89[A]	(3) [a] 159.77±48.89[A]	---
pooled	(18) 243.36±22.63	---	(11) 316.01±29.31	(15) 209.93±25.57	
			asparagine (μmol/L)		
0	---	(2) [a] 78.38±29.36	(1) [a] 48.78±41.52[A]	(3) [a] 65.22±23.97	---
12	(1) 146.62±41.52[A]	(1) 90.82±41.52	(3) 101.79±23.97[A]	(1) 58.18±41.52	(6) 99.35±18.95
24	(4) [a] 70.43±18.57[AB]	(2) [a] 90.81±29.36	(1) [a] 131.15±41.52[A]	---	---
60	(1) 34.78±41.52[B]	---	(2) 114.39±29.36[A]	(1) 87.38±41.52	---
pooled	---	---	(7) 99.03±17.47	---	
			cysteine (μmol/L)		
0	(6) [a] 82.70±12.12[A]	(3) [ab] 112.27±17.14[A]	(1) [ab]105.27±29.69[A]	(8) [b] 123.14±10.50[A]	(18) 108.34±9.46
12	(2) [a] 121.61±20.99[A]	(1) [a] 66.83±29.69[A]	(4) [a] 119.33±14.84[A]	(2) [a] 76.98±20.99[B]	(9) 96.19±11.13
24	(7) [a] 79.35±11.22[A]	(4) [ab] 114.73±14.84[A]	(3) [b] 144.91±17.14[A]	(2) [b] 155.97±20.99[A]	(16) 123.74±8.22
60	(3) [a] 80.58±17.14[A]	---	(3) [a] 89.18±17.14[A]	(3) [a] 118.04±17.14[AB]	---
pooled	(18) 91.06±7.63	---	(11) 114.67±10.28	(15) 118.53±8.96	
			glutamate (μmol/L)		
0	---	(2) [a] 17.16±12.00[A]	(1) [a] 21.99±16.97[A]	(4) [a] 30.21±8.48[A]	---
12	(1) [a] 27.38±16.97[A]	(1) [a] 17.44±16.97[A]	(3) [a] 46.36±9.80[A]	(2) [a] 33.87±12.00[A]	---
24	(6) [a] 27.24±6.93[A]	(3) [a] 34.97±9.80[A]	(1) [a] 57.22±16.97[A]	---	---
60	(2) 26.09±12.00[A]	---	(2) 37.43±12.00[A]	(2) 18.38±16.97[A]	---
pooled	---	---	---	---	
			glutamine (μmol/L)		
0	---	(2) [a] 2932.26±409.85[A]	(1) [a] 2376.13±579.62[A]	(4) [a] 2710.10±289.81[A]	---
12	(1) [a] 3245.75±579.62[A]	(1) [a] 3468.32±579.62[A]	(3) [a] 2536.17±334.64[A]	(2) [a] 3188.41±409.85[A]	---
24	(5) [a] 2808.68±259.21[A]	(3) [a] 2914.22±334.64[A]	(1) [a] 2374.33±579.62[A]	---	---
60	(2) [a] 2733.45±409.85[A]	---	(2) [a] 2650.74±409.85[A]	(2) [a] 1811.19±409.85[A]	---
pooled	---	---	---	---	
			glycine (μmol/L)		
0	(6) [a] 237.26±20.62[A]	(3) [a] 257.86±29.16[A]	(1) [a] 189.33±50.51[A]	(8) [a] 248.39±17.86[A]	(18) 233.21±16.10
12	(2) [a] 279.73±35.71[A]	(1) [a] 190.97±50.51[A]	(4) [a] 220.23±25.25[A]	(2) [a] 206.47±35.71[A]	(9) 224.35±18.94
24	(7) [a] 208.86±19.09[A]	(4) [ab]229.08±25.25[A]	(3) [ab] 273.64±29.16[A]	(2) [b] 318.87±35.71[A]	(16) 257.61±13.98
60	(3) [a] 229.36±29.16[A]	---	(3) [a] 174.14±29.16[A]	(3) [a] 278.46±29.16[A]	---
pooled	(18) 238.80±13.50	---	(11) 214.33±17.48	(15) 263.05±15.25	

Table 4. Continued

Dietary Glucose (%)	Days of gestation 18	19	20	21	Pooled
			histidine (μ mol/L) [2]		
0	(6) [a] 93.23±10.60[A]	(3) [ab] 123.23±9.18[A]	---	(8) [b] 180.34±30.27[A]	---
12	(2) [a] 120.13±8.00[A]	---	(4) [b] 179.64±8.03[A]	(2) [a] 76.22±28.27[B]	---
24	(7) [a] 94.62±9.68[A]	(4) [ab] 122.56±13.26[A]	(3) [ab] 174.32±19.68[A]	(2) [bc] 167.01±2.06[A]	(16) 139.63±6.43
60	(3) [a] !00.61±10.15[A]	---	(3) [a] 135.91±55.01[A]	(3) [a] 118.38±46.50[AB]	---
pooled	(18) 102.15±4.83	---	---	(15) 135.49±15.58	
			methionine (μmol/L)		
0	(6) [a] 231.92±18.44[A]	(3) [a] 238.18±26.08[A]	(1) [a] 167.19±45.17[A]	(8) [a] 218.91±15.97[A]	(18) 214.05±14.40
12	(2) [a] 285.26±31.94[A]	(1) [ab] 228.75±45.17[A]	(4) [b] 210.81±22.59[A]	(2) [ac] 262.46±31.94[AB]	(9) 246.82±16.94
24	(7) [a] 233.33±17.07[A]	(4) [a] 243.04±22.59[A]	(3) [a] 261.80±26.08[A]	(2) [a] 304.07±31.94[B]	(16) 260.56±12.51
60	(3) [a] 187.28±26.08[A]	---	(3) [a] 259.28±26.08[A]	(3) [bc] 298.22±26.08[B]	---
pooled	(18) 234.45±12.07	---	(11) 224.77±15.63	(15) 270.91±13.64	
			proline (μmol/L)		
0	(2) [a] 7192.50±8561.83[A]	(2) [a] 21756.5±8561.83[A]	(1) [a] 36813.0±12108.25[A]	(4) [a] 39623.25±6054.13[A]	(9) 26346.31±4540
12	(1) [a] 31509.0±12108.25[B]	(1) [a] 25317.0±12108.25[A]	(3) [a] 26165.33±6990.70[A]	(2) [a] 21252.5±8561.83[A]	(7) 26060±5095
24	(5) [a] 19296.8±5414.97[B]	(3) [a] 27126.67±6990.70 [A]	(1) [a] 28864.0±12108.25[A]	(1) [a] 12859.0±12108.25[A]	(10)22036.62
60	(2) 20931.0±8561.83[B]	---	(2) 28393.5±8561.83[A]	(1) 14905.0±12108.25[A]	---
pooled	(10) 19732.33±4489.86	---	(7) 30058.96±5095.31	(8) 22159.94±5019.82	
			serine (μmol/L)		
0	(4) 247.14±18.12[A]	---	---	(2) 223.43±25.63[A]	---
12	(1) [a] 225.46±36.25[A]	(1) [b] 240.80±36.25	---	(2) [c] 186.21±25.63[A]	---
24	(4) 217.75±18.12[A]	---	---	---	---
60	(1) 189.64 ±36.25[A]	---	(2) 254.71±25.63	(2) 234.89±25.63[A]	---
pooled	---	---	---	---	
			threonine (μmol/L)		
0	(4) 303.25±23.26[A]	---	---	(2) 277.22±32.89[A]	---
12	(1) 255.38±46.52[A]	(1) 272.74±46.52	---	(2) 207.26±32.89[A]	---
24	(4) 252.63±23.26[A]	---	---	---	---
60	(1) 237.82±46.52[A]	---	(2) 204.01±32.89	(2) 247.63±32.89[A]	---
pooled	---	---	---	---	
			valine (μmol/L)		
0	(6) [a] 259.09±16.23[A]	(3) [a] 251.77±22.96[A]	(1) [a] 190.16±39.77[A]	(8) [a] 229.07±14.06[AB]	(18) 232.52±12.67
12	(2) [a] 277.95±28.12[A]	(1) [a] 212.70±39.77[A]	(4) [a] 205.23±19.88[A]	(2) [a] 188.40±28.12[A]	(9) 221.07±14.91
24	(7) [a] 242.86±15.03[A]	(4) [a] 237.65±19.88[A]	(3) [a] 255.05±22.96[A]	(2) [a] 255.13±28.12[AB]	(16) 247.67±11.01
60	(3) [a] 228.66±22.96[A]	---	(3) [a] 183.06±22.96[A]	(3) [b] 290.37±22.96[B]	---
pooled	(18) 252.14±10.63	---	(11) 208.38±13.76	(15) 240.74±12.01	

[1] Values are least square means (LSM) ± SELSM with number of observations in parentheses. Values within a row with different lower case letters indicate significant differences between days of gestation ($p<0.05$); values within a column with different capital letters indicate significant differences between dietary glucose levels. [2] Values are weighted LSM ± SELSM.

Table 5. Amniotic fluid ketogenic amino acids [1]

Dietary Glucose (%)	Days of gestation 18	19	20	21	pooled
			leucine (μ mol/L)		
0	(6) [a] 300.92±24.79[A]	(3) [a] 315.10±35.06[A]	(1) [a] 242.68±60.73[A]	(8) [a] 278.09±21.47[AB]	(18) 284.20±19.35
12	(2) [a] 344.74±42.94[A]	(1) [b] 171.40±60.73[B]	(4) [ab] 256.59±30.36[A]	(2) [b] 179.75±42.94[A]	(9) 238.12±22.77
24	(7) [a] 262.36±22.95[A]	(4) [a] 271.99±30.36[AB]	(3) [a] 321.93±35.06[A]	(2) [a] 363.72±42.94[B]	(16) 305.00±16.81
60	(3) [a] 283.93±35.06[A]	—	(3) [a] 239.77±35.06[A]	(3) [a] 340.68±35.06[B]	—
pooled	(18) 297.99±16.23	—	(11) 265.24±21.02	(15) 290.56±18.33	
			lysine (μmol/L) [2]		
0	(6) [a] 297.36±17.29[A]	(3) [a] 287.77±9.18[A]	—	(8) [a] 273.55 ±11.32[A]	—
12	(2) [a] 303.59±6.79[A]	—	(4) [b] 247.62±12.37[A]	(2) [ab] 284.97±30.73[A]	—
24	(7) [ab] 280.89±13.89[A]	(4) [a] 260.14±11.58[A]	(3) [ab] 281.49±28.18[A]	(2) [b] 311.69±11.57[A]	(16) 283.55±8.86
60	(3) [a] 269.03±29.81[A]	—	(3) [a] 408.24±157.28[A]	(3) [a] 303.29±27.21[A]	—
pooled	(18) 287.72±9.44	—	—	(15) 293.38±11.03	

[1] Values are least square means (LSM) ± SELSM with number of observations in parentheses. Values within a row with different lower case letters indicate significant differences between days of gestation ($p<0.05$); values within a column with different capital letters indicate significant differences between dietary glucose levels. [2] Values are weighted LSM ± SELSM.

Table 6. Amniotic fluid ketogenic and glucogenic amino acids (listed in alphabetical order)[1]

Dietary Glucose (%)	Days of gestation 18	19	20	21	pooled
			isoleucine (μ mol/L)		
0	(6) ^{a} 127.13±21.11^{A}	(3) ^{ab} 158.30±29.86^{A}	(1) ^{ab} 252.31±51.71^{A}	(8) ^{b} 211.64±18.28^{A}	(18) 187.35±16.48
12	(2) ^{ab} 136.96±36.57^{A}	(1) ^{a} 64.96 ±51.71^{A}	(4) ^{b} 166.08±25.86^{A}	(2) ^{ac} 65.15±36.57^{B}	(9) 108.37±19.39
24	(7) ^{a} 100.52±19.55^{A}	(4) ^{a} 112.21±25.86^{A}	(3) ^{a} 145.07±29.86^{A}	(2) ^{a} 135.15±36.57^{AB}	(16) 123.24±14.32
60	(3) ^{a} 128.55±29.86^{A}	---	(3) ^{a} 135.20±29.86^{A}	(3) ^{a} 135.85±29.86^{AB}	---
pooled	(18) 123.29±13.82	---	(11) 174.66±17.90	(15) 137.03±15.61	
			phenylalanine (μmol/L)		
0	(6) ^{a} 222.28±29.74^{A}	(3) ^{a} 281.03±42.06^{A}	(1) ^{a} 321.60±72.84^{AB}	(8) ^{a} 305.89±25.75^{A}	(18) 282.70±23.21
12	(2) ^{a} 239.92±51.51^{A}	(1) ^{a} 149.59±72.84^{A}	(4) ^{b} 366.17±36.42^{AB}	(2) ^{a} 194.68±51.51^{A}	(9) 237.59±27.32
24	(7) ^{a} 202.48±27.53^{A}	(4) ^{a} 248.79±36.42^{A}	(3) ^{b} 477.12±42.06^{A}	(2) ^{b} 457.16±51.51^{B}	(16) 346.39±20.17
60	(3) ^{a} 201.90±42.06^{A}	---	(3) ^{a} 251.39±42.06^{B}	(3) ^{a} 324.37±42.06^{AB}	---
pooled	(18) 216.65±19.47	---	(11) 354.07±25.21	(15) 320.53±21.99	
			tryptophan (μmol/L) [2]		
0	(6) ^{a} 24.04±4.60^{A}	(3) ^{a} 33.84±6.51^{A}	(1) ^{a} 30.76±11.27^{A}	(8) ^{a} 32.95±3.98^{A}	(18) 30.14±3.59^{A}
12	(2) ^{a} 21.79±7.97^{AB}	(1) ^{a} 19.94±11.27^{A}	(4) ^{a} 36.09±5.63^{A}	(2) ^{a} 26.25±7.97^{AB}	(9) 25.33±4.23^{AB}
24	(7) ^{a} 14.51±4.26^{B}	(4) ^{ab} 26.85±5.63^{A}	(3) ^{b} 40.42±6.51^{A}	(2) ^{b} 44.54±7.97^{A}	(16) 28.94±3.12^{A}
60	(3) ^{a} 12.03±6.51^{B}	---	(2) ^{a} 19.02±7.97^{A}	(3) ^{a} 19.02±6.51^{B}	(8) 16.98±4.15^{B}
pooled	(10) ^{a} 17.39±3.01	(8) ^{ab} 24.22±4.53	(10) ^{b} 30.43±4.07	(15) ^{b} 29.25±3.40	
			tyrosine (μmol/L)		
0	(6) ^{a} 221.05±25.18^{A}	(3) ^{a} 248.11±35.61^{A}	(1) ^{a} 257.69±61.69^{AB}	(8) ^{a} 264.47±21.80^{A}	(18) 247.83±19.65
12	(2) ^{ab} 250.24±43.61^{A}	(1) ^{a} 154.33±61.67^{A}	(4) ^{b} 310.22±30.84^{AB}	(2) ^{ab} 233.03±43.61^{A}	(9) 236.96±23.13
24	(7) ^{a} 182.25±23.31^{A}	(4) ^{a} 214.92±30.84^{A}	(3) ^{b} 373.51±35.61^{A}	(2) ^{b} 455.61±43.61^{B}	(16) 306.58±17.07
60	(3) ^{a} 192.14±35.61^{A}	---	(3) ^{a} 193.43±35.61^{B}	(3) ^{a} 317.93±35.61^{AB}	---
pooled	(18) 211.42±16.48	---	(11) 283.71±21.35	(15) 317.76±18.62	

[1] Values are least square means (LSM) ± SELSM with number of observations in parentheses. Values within a row with different lower case letters indicate significant differences between days of gestation ($p<0.05$); values within a column with different capital letters indicate significant differences between dietary glucose levels. Pooled values within a row with different superscripts indicate significant differences between days of gestation ($p<0.05$); pooled values within a column with different superscripts indicate significant differences between dietary glucose levels ($p<0.05$). [2] Values are back transformed LSM (analysis done on log transformed data).

Table 7. Other amniotic fluid constistuents [1]

Dietary Glucose (%)	Days of gestation 18	19	20	21	pooled
			ethanolamine (μ mol/L)		
0	(6) [a] 22.20±2.70[A]	(3) [ab] 24.73±3.81[A]	(1) [ab] 36.64±6.61[A]	(8) [b] 30.85±2.34	(18) 28.60±2.11
12	(2) [a] 28.23±4.67[A]	(1) [a] 21.48±6.61[A]	(3) [a] 33.06±3.81[A]	(1) [a] 20.20±6.61	(7) 25.74±2.78
24	(7) [a] 19.64±2.50[A]	(4) [ab] 22.69±3.30[A]	(3) [b] 27.19±3.81[A]	(2) [b] 29.64±4.67	(16) 24.79±1.83
60	(3) [a] 22.71±3.81[A]	---	(3) [a] 20.03±3.81[A]	(3) [a] 28.09±3.81	---
pooled	(18) 23.19±1.77	---	(10) 29.23±2.34	(14) 27.19±2.31	
			γ-aminobutyrate (μmol/L)		
0	(6) [a] 3.86±1.89[A]	(3) [a] 2.53±2.67[A]	(1) [a] 0.0±4.62[A]	(8) [a] 4.41±1.63[A]	(18) 2.70±1.47
12	(2) [a] 6.09±3.27[A]	(1) [a] 5.42±4.62[A]	(4) [a] 4.06±2.31[A]	(2) [a] 2.98±3.27[A]	(9) 4.64±1.73
24	(7) [a] 5.78±1.75[A]	(4) [a] 2.64±3.31[A]	(3) [a] 2.49±2.67[A]	(2) [a] 2.31±3.27[A]	(16) 3.31±1.28
60	(3) [a] 5.95±2.67[A]	---	(3) [a] 6.18±2.67[A]	(3) [a] 6.03±2.67[A]	---
pooled	(18) 5.42±1.23	---	(11) 3.18±1.60	(15) 3.93±1.39	
			homocysteine (μmol/L)		
0	(6) [a] 4.27±2.23[A]	(3) [a] 8.68±3.15[A]	(1) [a] 5.79±5.45[A]	(8) [a] 6.83±1.93[AB]	(18) 6.39±1.74
12	(2) [a] 16.49±3.86[B]	(1) [a] 16.43±5.45[A]	(4) [a] 9.33±2.73[A]	(2) [a] 0.0±3.86[A]	(9) 10.56±2.05
24	(7) [a] 10.83±2.06[AB]	(4) [a] 6.22±2.73[A]	(3) [a] 10.76±3.15[A]	(2) [a] 5.35±3.86[AB]	(16) 8.29±1.51
60	(3) [a] 6.54±3.15[AB]	---	(3) [a] 5.35±3.15[A]	(3) [a] 8.22±3.15[B]	---
pooled	(18) 9.53±1.46	---	(11) 7.81±1.89	(15) 5.10±1.65	
			3-methylhistidine (μmol/L)		
0	(6) [a] 0.0±1.13	(3) [ab] 3.02±1.60[A]	(1) [bc] 9.95±2.78[A]	(8) [c] 12.41±0.98[A]	(18) 6.34±0.89
12	(2) [a] 0.0±1.96	(1) [ac] 0.0±2.78[A]	(4) [b] 7.95±1.39[A]	(2) [bc] 5.64±1.96[AB]	(9) 3.40±1.04
24	(7) [a] 0.0±1.05	(4) [a] 1.11±1.39[A]	(3) [b] 6.61±1.60[AB]	(2) [c] 9.97±1.96[AB]	(16) 4.42±0.77
60	(3) [a] 0.0±1.60	---	(3) [a] 1.80±1.60[B]	(3) [a] 3.94±1.60[B]	---
pooled	(18) 0.0±0.74	---	(11) 6.58±0.96	(15) 7.99±0.84	
			ornithine (μmol/L) [2]		
0	(6) [a] 38.05±3.60[A]	(3) [b] 49.18±0.59[A]	---	(8) [c] 96.92±17.62[A]	---
12	(2) [a] 34.91±1.78[A]	---	(4) [a] 55.35±3.46[A]	(2) [ab] 37.30±6.08[B]	---
24	(7) [a] 34.93±3.47[A]	(4) [a] 42.40±4.84[A]	(3) [ab] 69.14±15.40[A]	(2) [b] 58.52±1.98[A]	(16) 51.25±4.16
60	(3) [a] 36.09±4.15[A]	---	(3) [a] 37.62±9.20[A]	(3) [a] 41.73±17.70[AB]	---
pooled	(18) 36.00±1.68	---	---	(15) 58.62±6.45	
			phosphoserine (μmol/L)		
0	(6) [a] 3.69±0.50[A]	(3) [ab] 4.27±0.71[A]	(1) [ab] 6.07±1.23[A]	(8) [b] 5.97±0.44[A]	(18) 5.00±0.39
12	(2) [a] 4.99±0.87[B]	(1) [a] 4.06±1.23[A]	(4) [a] 5.92±0.62[A]	(2) [a] 5.18±0.87[A]	(9) 5.04±0.46
24	(7) [a] 3.83±0.47[AB]	(4) [ab] 5.02±0.62[A]	(3) [b] 5.99±0.71[A]	(2) [b] 5.90±0.87[A]	(16) 5.19±0.34
60	(3) [a] 4.10±0.71[AB]	---	(2) [a] 5.77±0.87[A]	(3) [a] 4.18±0.71[A]	---
pooled	(18) 4.15±0.33	---	(10) 5.94±0.45	(15) 5.31±0.37	
			taurine (μmol/L)		
0	(6) [a] 178.77±13.89[A]	(3) [ab] 203.39±19.65[A]	(1) [ab] 242.48±34.04[AB]	(8) [b] 258.04±12.03[A]	(18) 220.67±10.85
12	(2) [ab] 173.65±24.07[A]	(1) [ab] 206.61±34.04[A]	(4) [a] 223.30±17.02[A]	(2) [b] 105.30±24.07[B]	(9) 177.22±12.76
24	(7) [a] 155.14±12.86[A]	(4) [bd] 215.12±17.02[A]	(3) [cd] 249.89±19.65[A]	(2) [ab] 193.08±24.07[AB]	(16) 203.31±9.42
60	(3) [a] 153.27±19.65[A]	---	(3) [a] 151.90±19.65[B]	(3) [a] 127.58±19.65[B]	---
pooled	(18) 165.21±9.10	---	(11) 216.89±11.78	(15) 171.00±10.28	

[1] Values are least square means (LSM) ± SELSM with number of observations in parentheses. Values within a row with different lower case letters indicate significant differences between days of gestation ($p<0.05$); values within a column with different capital letters indicate significant differences between dietary glucose levels. [2] Values are weighted LSM ± SELSM.

Table 8. Pearson correlation coefficients of amniotic fluid amino acids with fetal growth parameters[1]

Amniotic fluid amino acids	(n)	Fetal WEIGHT	Fetal LUNG WEIGHT	Fetal HEART WEIGHT
glucogenic				
alanine	51	0.118	0.190	0.112
arginine	51	0.097	0.059	0.064
asparagine	23	0.183	0.180	-0.306
cysteine	51	0.489***	0.425**	0.299*
glutamate	28	0.330	0.328	0.291
glutamine	28	-0.359	-0.368	-0.199
glycine	51	0.218	0.141	0.157
histidine	51	0.455***	0.398**	0.369**
methionine	51	0.341*	0.371**	0.168
proline	30	0.209	0.200	0.414*
serine	18	-0.58	0.101	0.074
threonine	18	-0.290	-0.332	-0.066
valine	51	-0.072	-0.089	-0.080
ketogenic				
leucine	51	0.094	0.110	0.065
lysine	52	0.073	0.119	0.158
ketogenic+glucogenic				
isoleucine	51	0.261	0.160	0.229
phenylalanine	51	0.569****	0.537****	0.363**
tryptophan	51	0.406**	0.375**	0.377**
tyrosine	51	0.558****	0.483***	0.377**
other				
ethanolamine	49	0.332*	0.218	0.276
γ-aminobutyrate	51	-0.049	-0.061	-0.181
homocysteine	51	-0.197	-0.139	-0.199
3-methylhistidine	51	0.639****	0.460***	0.629****
ornithine	52	0.375**	0.262	0.535****
phosphoserine	50	0.515****	0.488***	0.441**
taurine	51	0.161	0.137	0.354*

[1] Probability of Pearson correlation coefficient: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Table 9. Pearson correlation coefficients of amniotic fluid amino acids with fetal tissue glycogen reserves [1]

Amniotic fluid amino acids	Fetal					
	(n)	LIVER GLYCOGEN	(n)	LUNG GLYCOGEN	(n)	HEART GLYCOGEN
Glucogenic						
Alanine	50	0.166	51	0.170	51	0.222
Arginine	50	-0.074	51	0.355*	51	-0.011
Asparagine	22	0.146	23	0.557**	23	0.360
Cysteine	50	0.293*	51	0.320*	51	0.231
Glutamate	27	0.213	28	0.272	28	0.109
Glutamine	27	-0.614***	28	0.050	28	-0.189
Glycine	50	0.213	51	0.154	51	0.096
Histidine	50	0.198	51	0.228	51	0.124
Methionine	50	0.351*	51	0.224	51	0.393**
Proline	29	-0.146	30	0.233	30	-0.106
Serine	17	-0.131	18	0.447	18	-0.080
Threonine	17	-0.305	18	-0.018	18	-0.250
Valine	50	0.027	51	0.002	51	0.041
ketogenic						
Leucine	50	0.116	51	0.079	51	0.031
Lysine	51	0.106	52	0.010	52	0.106
Ketogenic+glucogenic						
Isoleucine	50	-0.033	51	0.077	51	-0.027
Phenylalanine	50	0.475***	51	0.437**	51	0.262
Tryptophan	50	0.224	51	0.326*	51	0.121
Tyrosine	50	0.505***	51	0.327*	51	0.275
other						
Ethanolamine	48	0.077	49	0.176	49	-0.023
γ-aminobutyrate	50	-0.043	51	-0.036	51	0.083
Homocysteine	50	-0.099	51	0.246	51	-0.070
3-methylhistidine	50	0.324*	51	0.028	51	0.034
Ornithine	50	0.020	52	-0.033	52	-0.108
Phosphoserine	49	0.184	50	0.168	50	0.089
Taurine	50	-0.161	51	0.123	51	-0.333*

[1] Probability of Pearson correlation coefficient: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Table 10. Amniotc fluid constituents as predictors of fetal growth parameters using multiple regression[1]

Amniotic fluid Constituents	Fetal		
	WEIGHT (n=47)	LUNG WEIGHT (n=49)	HEART WEIGHT (n=49)
Glucose	-0.009 (0.008)	-0.065 (0.27)	-0.020 (0.07)
Glucogenic			
Cysteine	-0.010 (0.008)	-0.600 (0.28)*	-0.023 (0.06)
Histidine	0.007 (0.006)	-0.067 (0.16)	-0.080 (0.05)
Methionine	0.011 (0.005)*	0.434 (0.15)**	---
Ketogenic+glucogenic			
Phenylalanine	0.006 (0.004)	0.383 (0.15)*	-0.011 (0.04)
Tryptophan	-0.021 (0.01)	-0.471 (0.51)	-0.037 (0.08)
Tyrosine	-0.003 (0.004)	-0.200 (0.16)	0.053 (0.04)
other			
Ethanolamine	-0.019 (0.02)	---	---
3-methylhistidine	0.160 (0.05)**	2.85 (1.54)	0.740 (0.37)
Ornithine	-0.013 (0.009)	---	0.153 (0.08)
Phosphoserine	0.076 (0.15)	7.34 (5.36)	0.467 (1.30)
Taurine	---	---	-0.004 (0.03)
F-value	7.09****	4.80***	4.40***
Adjusted Mult. R^2	0.59	0.41	0.41
Intercept	0.67 (0.90)	-31.94 (29.13)	24.97 (6.91)***

[1] Values are parameter estimates (SEM). Probability: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

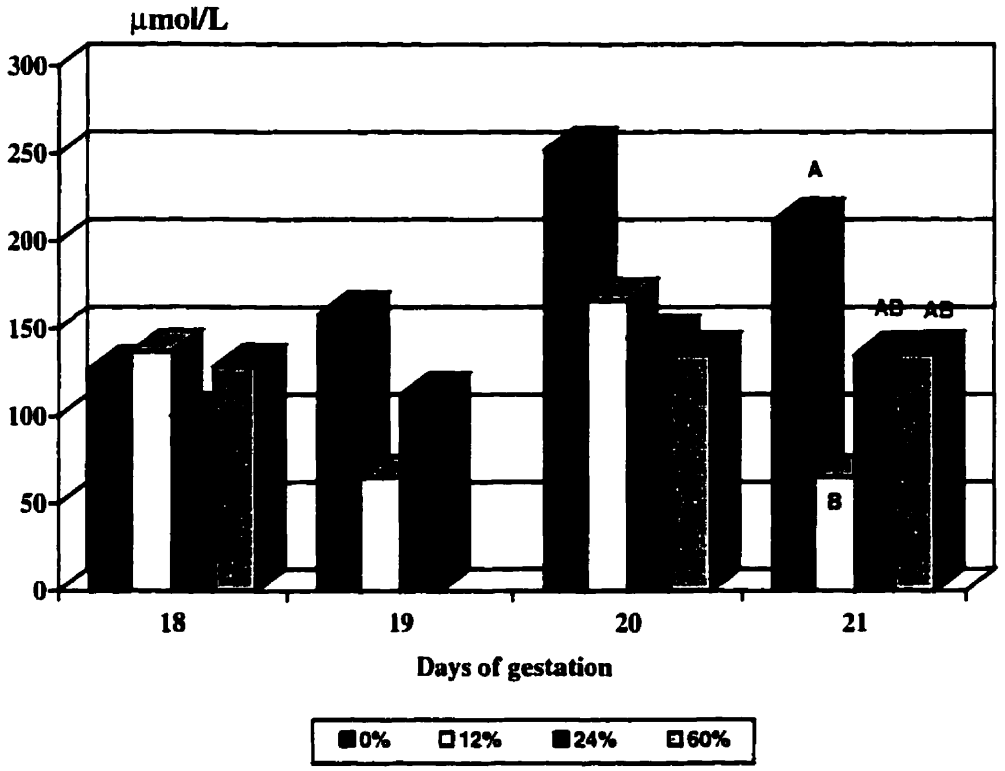
Table 11. Amniotc fluid constituents as predictors of fetal tissue glycogen reserves using multiple regression[1]

Amniotic fluid constituents	Fetal		
	LIVER GLYCOGEN (n=49)	LUNG GLYCOGEN (n=49)	HEART GLYCOGEN (n=50)
glucose	---	0.099 (0.03)***	---
glucogenic			
arginine	---	0.004 (0.01)	---
cysteine	-0.404 (0.12)**	-0.003 (0.03)	---
methionine	0.200 (0.06)**	0.009 (0.02)	0.022 (0.006)**
ketogenic+glucogenic			
phenylalanine	0.091 (0.06)	0.041 (0.02)*	---
tryptophan	---	0.068 (0.07)	---
tyrosine	0.043 (0.07)	-0.031 (0.02)	---
other			
3-methylhistidine	1.11 (0.50)*	---	---
taurine	---	---	-0.018 (0.007)**
F-value	7.149****	5.469***	8.686***
Adjusted Mult. R^2	0.39	0.35	0.24
Intercept	-33.30 (10.22)**	0.59 (2.47)	4.84 (2.13)*

[1] Values are parameter estimates (SEM). Probability: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Figure 1. The effect of a) dietary glucose levels and b) days of gestation on amniotic fluid isoleucine concentration.

a)



b)

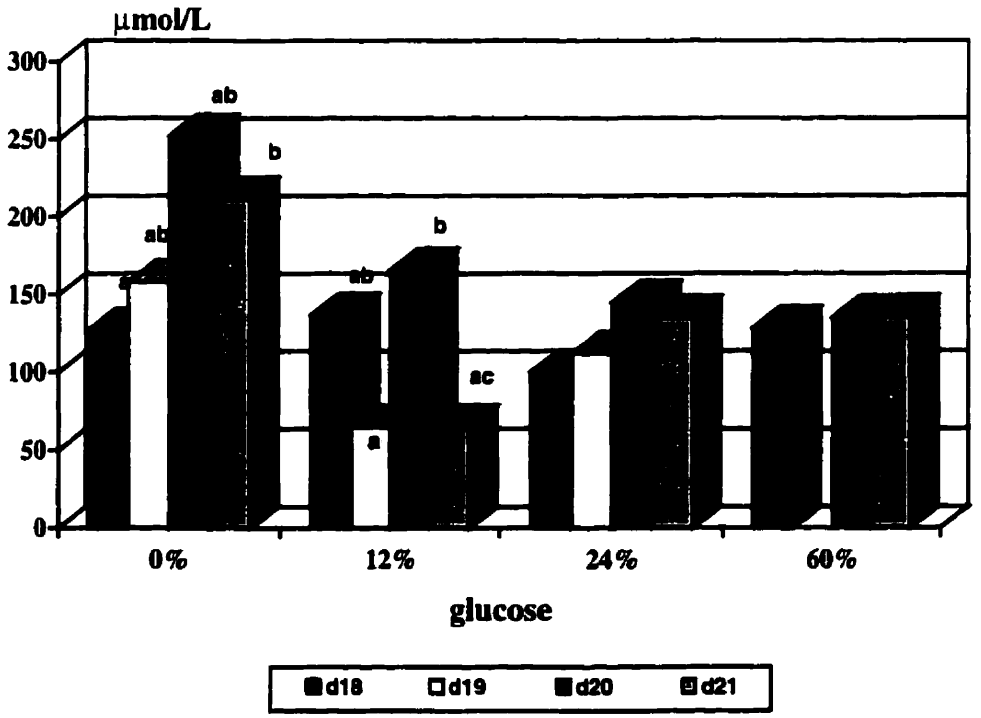
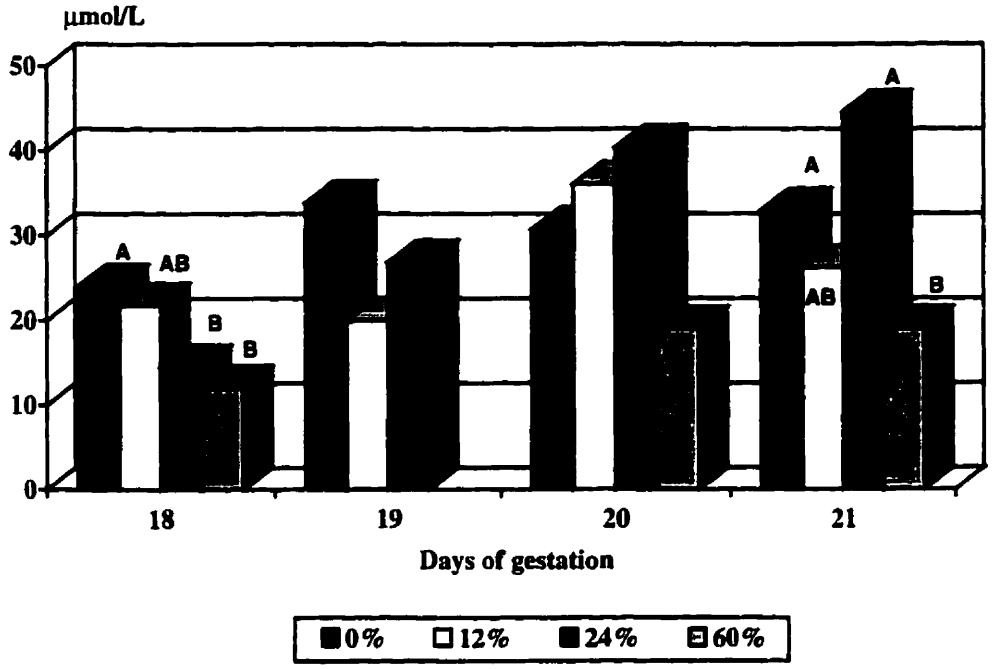


Figure 2. The effect of a) dietary glucose levels and b) days of gestation on amniotic fluid tryptophan concentration.

a)



b)

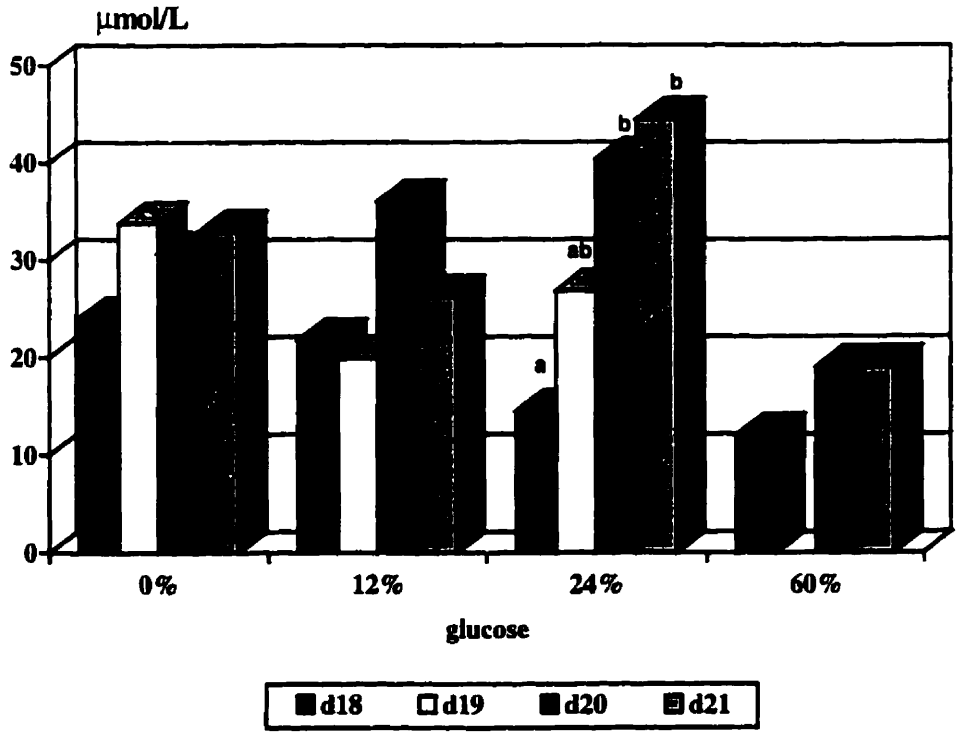
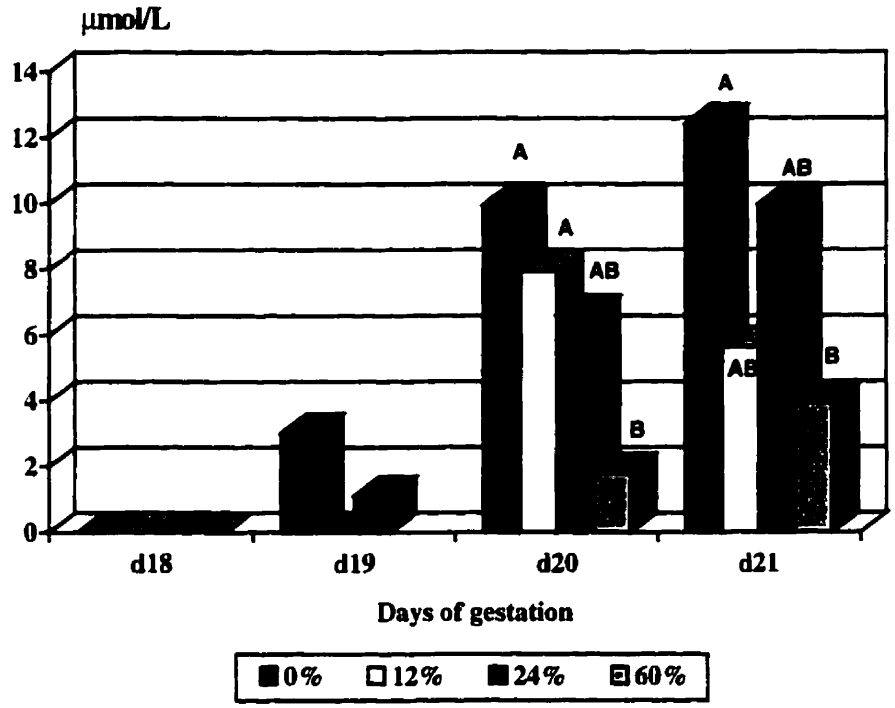


Figure 3. The effect of a) dietary glucose levels and b) gestational days on amniotic fluid 3-methylhistidine concentration.

a)



b)

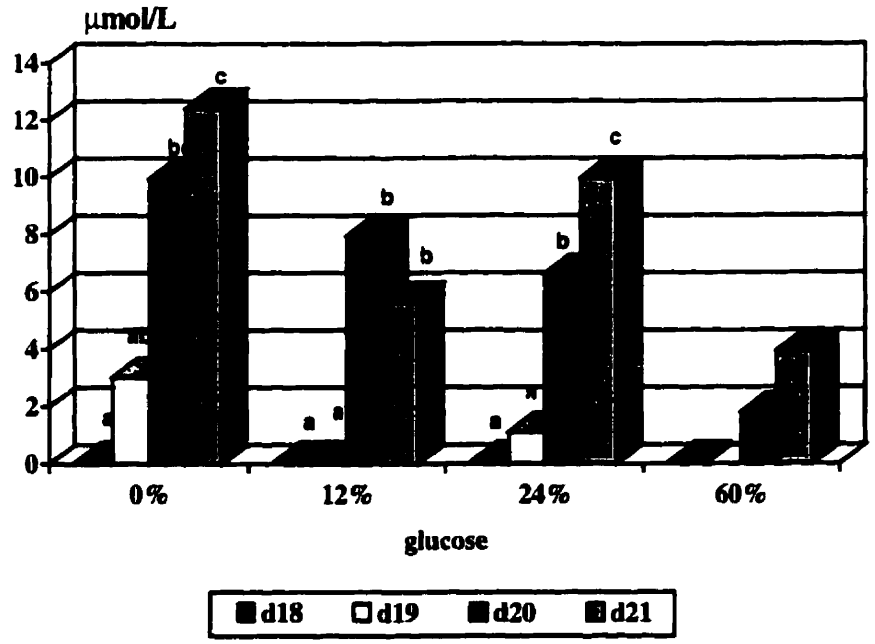
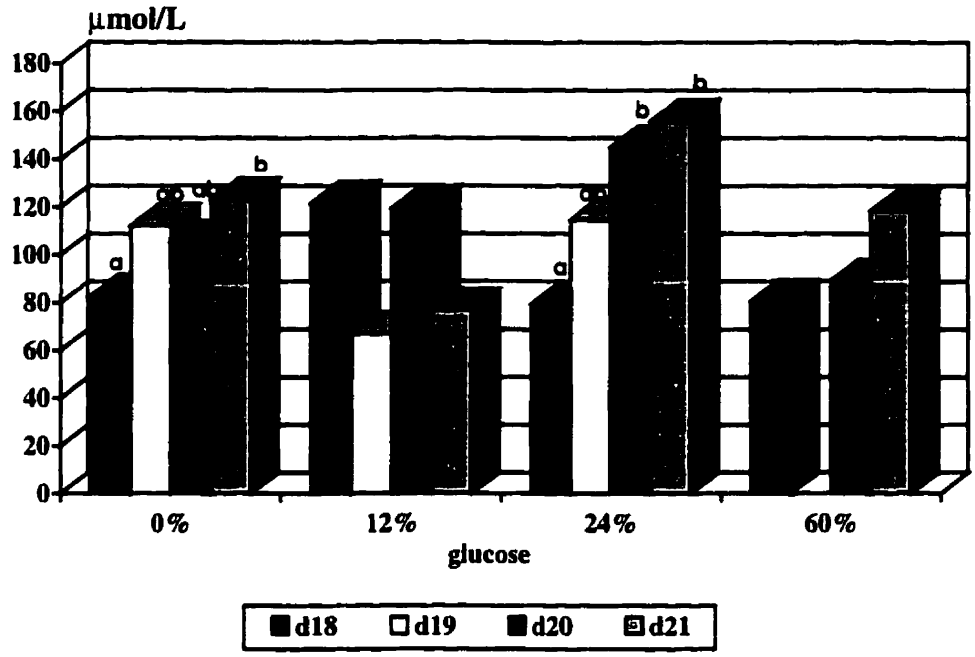
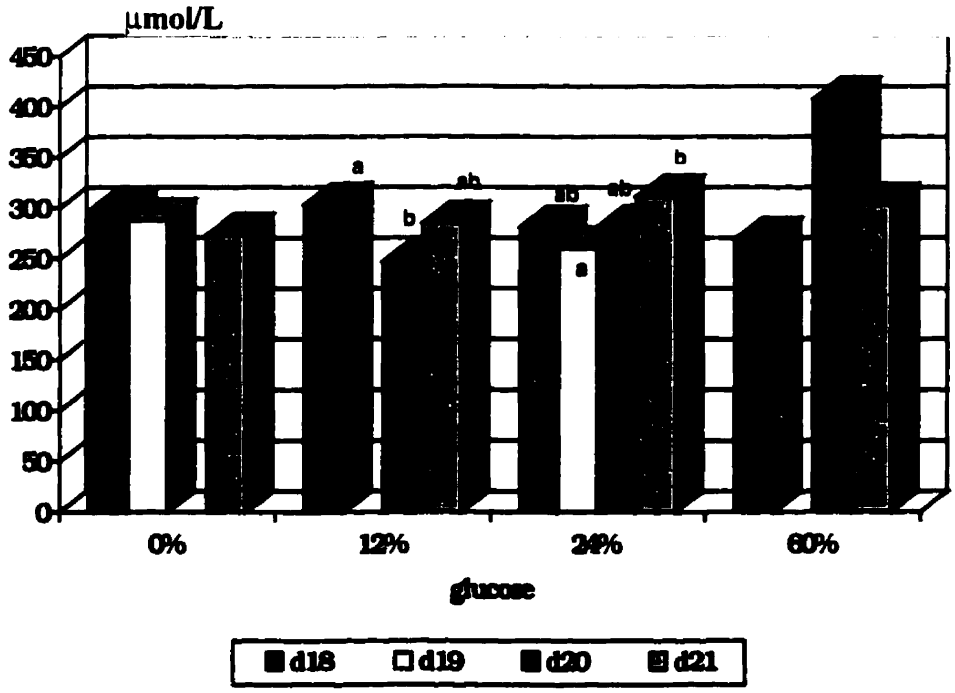


Figure 4. The effect of gestational days on:

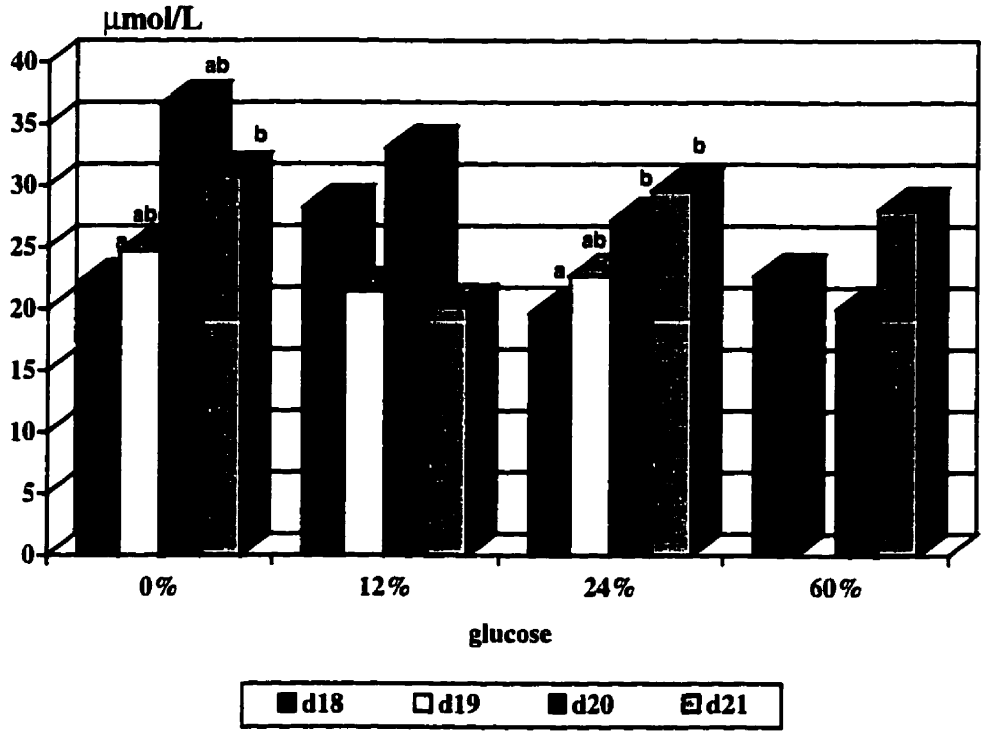
a) cysteine



b) lysine



c) ethanolamine



d) phosphoserine

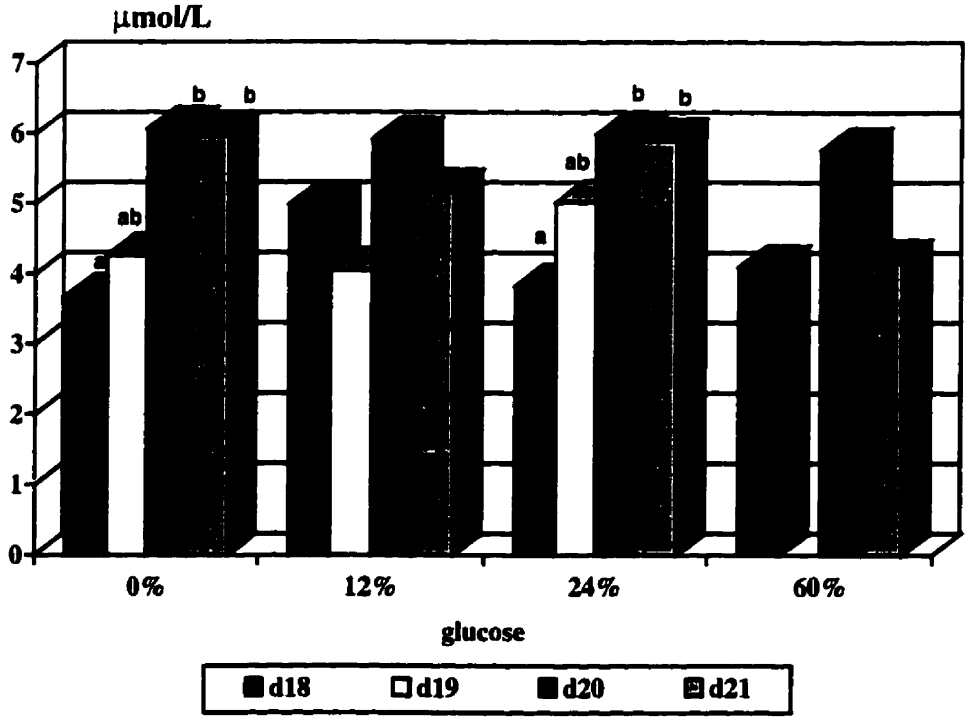
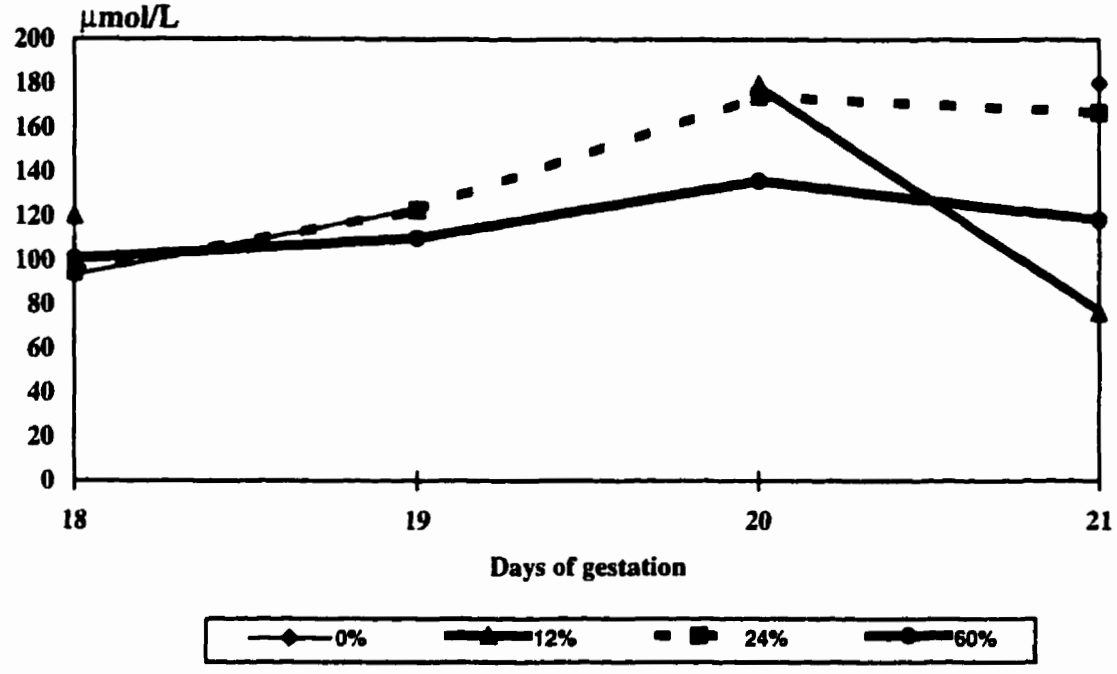
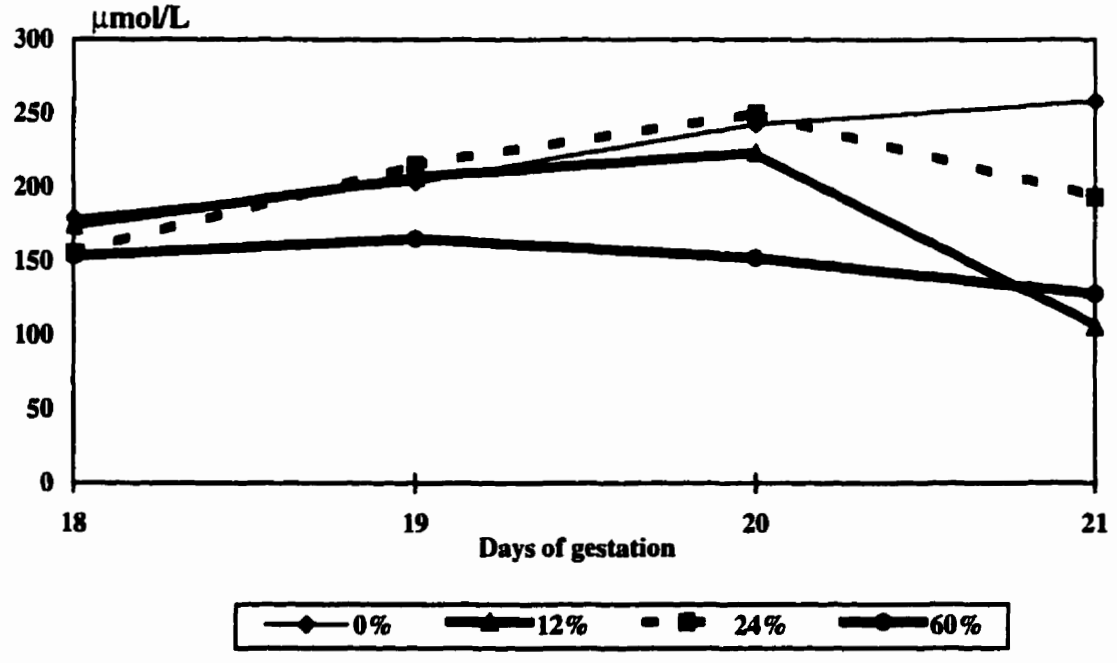


Figure 5. Interaction effect between dietary glucose and day of gestation on amf histidine and taurine concentrations.

a) histidine



b) taurine



CHAPTER 4 - DISCUSSION

This study was the first to report on the consequences of reduced maternal dietary glucose supply on the profile of amniotic fluid (amf) amino acids in rats during the last four days of gestation (gd 18.5 to 21.5). The use of isocaloric diets containing graded levels of glucose revealed that *(1)* maternal dietary glucose restriction significantly increased the concentrations of amf isoleucine (on gd 21.5), tryptophan (on gd 18.5 and 21.5) and 3-methylhistidine (on gd 20.5 and 21.5) but had no effect on the principal glucogenic amino acids alanine and glycine and the branched-chain amino acids (BCAA) valine and leucine, *(2)* an interaction between diet and day of gestation modified amf taurine levels such that dams fed low carbohydrate diets showed significant increases in amf taurine as pregnancy progressed, *(3)* the examination of amf amino acids at different time points (gd 18.5 to 21.5) provided evidence that gestational period plays a critical role in determining the concentration of certain amf amino acids, *(4)* specific amf amino acids correlated with fetal growth parameters and fetal tissue glycogen reserves indicating the ability of amf composition to reflect fetal distress under conditions of compromised maternal nutritional status and *(5)* using amf constituents as predictors of fetal growth parameters and fetal tissue glycogen reserves, we found fetal growth , and not fetal glycogen reserves to be predicted by specific amf amino acids.

Complete maternal dietary glucose restriction (0%) fed throughout pregnancy resulted in a significant increase in the amf concentration of the BCAA, isoleucine

on day 21.5 of gestation in our study. Only two other studies have reported the response of amf isoleucine to nutritional deprivation. Similar results were found by Felig and coworkers (1972), who reported that after a period of 84-90 hours of maternal starvation, during weeks 16-22 of gestation, amf concentration of isoleucine increased. However, contrasting results were observed by Bernstein and coworkers (1992), who reported a significant decrease in amf isoleucine in rats fasted for 72 hours during days 18.5 and 21.5 of gestation. Explanation for this difference may be related to the severity, length and timing of the insult. The study by Bernstein and coworkers (1992) looked at short-term maternal starvation in term gestation (gd 18.5 to 21.5) when fetal protein accretion is most rapid. In contrast, our study provided dams with isocaloric diets deficient in a specific macronutrient, glucose, which was fed throughout gestation. Our finding of an increase in amf isoleucine, in the absence of exogenous glucose intake, supports the metabolic adaptations that normally occur to maintain glucose homeostasis. The increase in this BCAA reflects an increase in protein catabolism, which provides the necessary nitrogens and carbon skeletons required for the synthesis of alanine, thereby increasing substrate availability for gluconeogenesis. This accelerated oxidation of isoleucine in muscle tissue is further supported by the results from two separate studies by our laboratory (Koski and Fergusson, 1992; Lanoue, 1993) who report an increase in amf urea and uric acid concentrations in dams fed carbohydrate-restricted diets. This increase in amf urea and uric acid is suggestive of increased muscle protein turnover.

This is the first study to report on amf tryptophan levels with altered maternal nutritional status. In early gestation, dams fed 0% glucose diets had significantly higher amf tryptophan levels than dams fed 24% and 60% glucose diets. In term fetuses, amf tryptophan levels rose significantly in dams fed 0% and 24% glucose diets as compared to control dams (60%). Tryptophan, an essential amino acid, is one of the first amino acids which maintains nitrogen equilibrium to compensate for the metabolic turnover of body protein (Borg and Wahlstrom, 1989). Tryptophan may have been diverted from protein synthesis and accretion to protein catabolism and oxidation, a process that compromises fetal growth. This may explain the detection of high amf tryptophan levels in dams that were deprived of dietary glucose.

The urinary excretion of 3-methylhistidine has been proposed to be a reliable index of the rate of muscle protein breakdown (Lukaski et al, 1981). However, it is important to note that several factors may invalidate this index. The effects of factors such as sex, age, nutritional status, hormonal status, injury and disease have yet to be quantified (Buskirk and Mendez, 1985). In addition, any catabolic state such as fever, starvation, trauma and infection can increase the muscle turnover rate and alter the relationship between muscle mass and urinary excretion of 3-methylhistidine (Gibson, 1990). The higher 3-methylhistidine concentrations which were observed in the amf of dams fed the 0% and 12% glucose diets on day 20.5 of gestation and in dams fed the 0% glucose diets at term, compared with the control diet (60%), suggests that in the absence of adequate glucose supply there is an increase in the protein turnover rate of fetal muscle. This is reflected by the increase in 3-

methylhistidine detected in amf, although we are not able to tell whether 3-methylhistidine was of fetal or maternal origin. In addition, because 3-methylhistidine can also originate from the gut and fetal muscle mass is quite low in the fetus, it becomes difficult to interprete these present results. It is known that fetal urine becomes the primary source of amf towards the end of gestation (Wallenburg, 1977, Van Otterlo et al, 1977) with increasing maturation of fetal renal tubular function. This may also explain why we did not detect any 3-methylhistidine in the amf of gd 18.5 fetuses and very little in day 19.5 fetuses, which would reflect therefore the immaturity of the fetal renal tubular function at this point of gestation.

No other study has reported on the effect of compromised maternal nutritional status on the concentration of amf taurine. Our results show that an interaction between diet and day of gestation modified amf taurine levels such that dams fed low carbohydrate diets showed significant increases in amf taurine as pregnancy progressed. In addition, the difference between dietary groups (0% and 24%) was greater during gestational days 20.5 and 21.5 than at gestational days 18.5 and 19.5.

The amf concentrations of the principle glucogenic precursors alanine and glycine and the BCAA valine and leucine have been reported to show distinct responses to maternal substrate deprivation. This is evident in two separate studies in which Felig and coworkers (1972) reported a significant decrease in amf alanine and significant increases in the BCAA valine and leucine following 72 hours of maternal starvation during the second trimester of pregnancy. Similarly, Tyson and coworkers

(1976), observed a 30% fall in amf alanine and an 18% fall in amf glycine in women undergoing 72 hours of fasting in the first half of gestation. Bernstein and coworkers (1992) found that amf alanine and glycine decreased in rats fasted for 72 hours in term gestation. In general, acute fasting appears to produce significant alterations in carbohydrate metabolism which is accomplished by the utilization of glucogenic amino acids, principally alanine, and that this amino acid is more rapidly depleted during fasting in pregnancy than in non pregnant animals (Felig et al, 1972). However, no changes in amf alanine, glycine and the BCAA valine and leucine were observed in our study. The reason for this discrepancy between our dietary model and that of fasting may be that unlike fasting, our diets, although deficient in glucose, met the energy and protein requirements for pregnant dams (NRC 1978) and therefore may have not provided a severe enough dietary insult to perturb the concentrations of amf alanine, glycine, valine and leucine. It is also possible that these amino acids are more affected by a lack of dietary energy or another nutrient than of glucose. Although our sample size was larger than those reported by these previous studies, we would suggest that a larger sample size might result in statistical significance of these amf amino acids with this same dietary model.

This study brings forth the importance of the stage of gestation in determining the concentration of amf amino acids. Previous studies have shown that the profile of amf amino acids is species specific and dependent on the stage of pregnancy under study. In humans, the concentration of amf amino acids have been shown to decrease with the progression of pregnancy, with the exception of taurine

(Emery and Scrimgeour, 1970; O'Neill et al, 1971; Velazquez et al, 1976). In rats, however, the concentration of most amf amino acids increase, particularly between days 17 to 20 of gestation (Wirtschafter, 1958; McEvoy-Bowe et al, 1987). Our results show that amf concentrations are dependent on the stage of pregnancy, which is reflected by the fact that 12 of the 26 amf amino acids analyzed significantly responded to the main effect of gestational day. Significance was found with amf cysteine, histidine, lysine, isoleucine, phenylalanine, tryptophan, tyrosine, ethanolamine, 3-methylhistidine, ornithine, phosphoserine and taurine. Of these amino acids, amf cysteine, isoleucine, tryptophan, ethanolamine, 3-methylhistidine and phosphoserine increased with the progression of pregnancy in those dams fed glucose-restricted diets. No clear biological pattern could be described for the remainder of the amino acids. No significant difference in the concentration of amf amino acids with day of gestation was found in dams fed the 60% control diets. We can conclude that the stage of gestation plays a critical role in determining the concentration of amf amino acids but we do recognize that this is driven, in part, by the fact that our animal model is one with a short gestational period. This presents a situation in which changes in amf amino acid concentrations occur quite rapidly. Amf sampling was taken during the period when fetal rats are undergoing rapid growth (gd 18.5 to 21.5). With an animal model of longer gestation, amf sampling can be taken over longer intervals, minimizing the rapid daily fluctuations of amf amino acids that occur during the period of most rapid fetal growth. In addition, for some of these amino acids, namely, isoleucine, cysteine, lysine, ethanolamine,

phosphoserine and 3-methylhistidine, our conclusions are limited by a small sample size. We do recognize that our conclusions for these amf amino acids were based on observations of sample size of N=1. This was due to technical difficulties we encountered with the amino acid analyzer. Therefore a larger sample size could also clarify future findings.

Our results showed a statistical interaction between diet and day of gestation with the concentration of amf histidine, leucine, phenylalanine, tyrosine and ornithine. Although a statistical significance was obtained with these amino acids, it is difficult to describe any biological pattern as a result of dietary treatment during the last four days of gestation. The fluctuation of these amino acids may be due to the use of a short gestational animal model. A different animal species with a longer gestational period would possibly eliminate the rapid fluctuations that occur within such a short gestational period when fetal growth is most rapid. In addition, sample size may also be a limiting factor as some of the observations are based on a small sample size. Therefore increasing sample size would validate future findings.

One of the more important contributions of this study was in demonstrating the sensitivity of the amf compartment to a maternal dietary deficiency of just a single macronutrient, glucose. This ability of the amf composition to respond to maternal diet offers promise in the use of amf to diagnose maternal and fetal nutritional status. Key amf amino acids (isoleucine, tryptophan, 3-methylhistidine and taurine) were altered by dietary glucose restrictions and these amino acids are often those which are used to explain the metabolic adaptations which occur to

maintain glucose homeostasis, normally in response to maternal starvation (Meschia, 1978; Johnson et al, 1986). This study also suggests the importance of amf analysis during specific gestational periods. It was found that the concentrations of certain amf amino acids are highly dependent on the stage of pregnancy, and amf sampling will vary accordingly.

Even though amf composition has been used to monitor fetal maturity, noting that lowered amf glucose was associated with fetal growth retardation (Drazancic and Kuvacic, 1974), few studies have examined the possibility of using this pool in predicting fetal metabolic distress during compromised maternal nutritional status. Two separate reports from our laboratory, using a similar dietary model, describe amf constituents (glucose, urea and uric acid) as sensitive indicators of fetal distress in response to maternal dietary glucose deprivation (Koski and Fergusson, 1992; Lanoue, 1993). In the study by Koski and Fergusson (1992), the results showed that with maternal-carbohydrate restriction, higher concentrations of amf glucose were correlated with increased fetal weight and liver glycogen deposition. In addition, Lanoue (1993) found that higher concentrations of amf glucose were also correlated with increased fetal heart and lung glycogen reserves. Koski and Fergusson (1992) reported that amf urea and uric acid were negatively correlated with fetal weight and liver glycogen reserves. However, these authors found that amf uric acid responded with significant measurable changes over the entire range of maternal dietary carbohydrate intake whereas amf urea was less sensitive showing only significantly greater levels in rats fed low (0%) carbohydrate diets. Lanoue (1993) found that amf

urea was negatively correlated with fetal heart glycogen reserves only and not with any other fetal metabolic measurements. In both these studies, the authors concluded that amf glucose was a better indicator of fetal metabolic distress than amf urea nitrogen. Their results indicate the ability of amf composition to reflect fetal distress with compromised maternal nutritional status. Our results reinforce this concept and are the first to show that specific amf amino acids correlate with fetal growth parameters and fetal tissue glycogen reserves in response to maternal dietary glucose restriction. Amf cysteine, histidine, phenylalanine, tryptophan, tyrosine, 3-methylhistidine and phosphoserine were positively correlated with all three growth parameters, fetal weight, lung weight and heart weight. Whereas amf proline and taurine were positively correlated with fetal heart weight, amf ethanolamine was positively correlated with fetal weight only. Not as many amf amino acids were correlated with fetal glycogen reserves. These correlation data suggests that measurements of these amino acids might be better predictors of fetal growth.

Regression analysis showed that amf methionine and 3-methylhistidine were predictors of fetal weight and explained 59% of its variability. Lanoue (1993) found that amf glucose was not a valid predictor of fetal weight in dams fed carbohydrate-restricted diets. In support of this, our results showed that when amf glucose was included in our regression model, it was found that it was not a significant predictor of fetal weight nor of fetal lung weight or fetal heart weight. This suggests that amf amino acids may be better predictors of fetal growth status than amf glucose during maternal dietary glucose deprivation. However, our results showed that amf amino

acids were not found to be predictors of fetal heart weight. Furthermore, we found that amf amino acids when combined with glucose in the same statistical model were less sensitive predictors of fetal tissue glycogen reserves. This stems from the observation that the regression model for fetal liver, lung and heart glycogen explained 39%, 35% and 24% of their variability, respectively. Amf glucose was found to be correlated with fetal liver, lung and heart glycogen reserves (Koski and Fergusson, 1992; Lanoue, 1993). However, contrasting results were obtained in our study, which shows that when amino acids and glucose were both included in the regression model, amf glucose was found to be correlated and a predictor of fetal lung glycogen only. Amf glucose may be more important to fetal lung glycogen deposition as it coincides with peak lung glycogen deposition which occurs on days 19-20 of gestation in the fetal rat (Bourbon and Jost, 1982, Maniscalco et al, 1978). This illustrates the importance of having specific amf constituents on particular gestational days. It also brings forth the possibility that, depending on the day of gestation, different amf constituents may be predictors of fetal growth and metabolic status depending on their requirement for fetal growth. In this study, we did not have sufficient sample size to perform stepwise regression by day of development. We would have to increase the sample size to perform this statistical analysis to determine whether gestational day influences the predictability of amf constituents. Our result also showed that amf taurine, an essential amino acid for fetal growth, was a negative predictor of fetal heart glycogen only.

In conclusion, this study further emphasizes that dietary glucose is essential for fetal growth and development (Jones and Rolph, 1985; Koski and Hill, 1990). In this diet-induced model of fetal distress, lack of maternal dietary glucose brings about metabolic adaptations that are reflected as alterations in the concentrations of amf amino acids. It is known that the amf pool represents a nutritional reservoir for the developing fetus and contributes in part to fetal growth (Mulvihill et al, 1985). Therefore it follows that any alterations in the composition of amf can have detrimental effects on fetal growth, In this study elevated levels of isoleucine, tryptophan, 3-methylhistidine and taurine were found in the growth retarded fetuses of dams fed glucose restricted diets. However, this information does not allow us to determine the origin of these amf amino acids. Since the amf is a composite of maternal, fetal and placental circulation, future research should include amino acid tracer studies. This would allow for the determination of the origin of amf amino acids and to further understand the maternal, fetal and placental metabolic adaptations to maternal nutrient deficiency. This study also highlights the dependence of amf amino acids to the gestational period under study. Our data is suggestive that depending on the gestational period, different amf amino acids may be predictors of fetal growth and metabolic status. However, we feel our study did not have a sufficient sample size to clarify this issue given the short gestation of the rat. Another difficulty that was evident in this study, was that with a short gestational animal model the concentrations of amf amino acids are quickly changing. Our measurements were obtained during the period when fetal growth is most rapid

(gd18-21), making the interpretation of amf amino acids more difficult since a few hours could represent a significant change in the amf compartment. This could be resolved by using an animal model with a longer gestational period and with more of the fetal growth occurring in utero. Future research should include the analysis of other extracellular fluid amino acid pools, specifically maternal and fetal plasma pools. The comparison between maternal and fetal plasma and amf amino acid pools would provide additional information for assessing metabolic status and to determine whether any changes are reflected in a similar fashion in each of these compartments.

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