IN VIVO TRANSPLACENTAL TRANSPORT AND METABOLISM IN SMALL AND APPROPRIATE FOR GESTATIONAL AGE FETUSES

A thesis submitted for the degree of

DOCTOR OF MEDICINE (University of Leicester)

by Justin Chi Konje MBBS, FWACS, FMCOG, MRCOG

Department of Obstetrics & Gynaecology
University of Leicester Medical School
Robert Kilpatrick Clinical Sciences Building
Leicester, LE2 7LX

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Abstract

Being small for gestational age (SGA) is associated with a higher perinatal morbidity and mortality, neurodevelopmental disability and various adult diseases. The concentration of some amino acids such as leucine, lysine, glycine and phenylalanine has been shown to be reduced in the plasma of SGA fetuses. These reductions could be due to defective transport across the placenta, diminished uptake or increased metabolism in the fetus. The studies described in this thesis were designed to test the hypotheses that the reduced concentration of two of these amino acids (leucine and glycine) is due partly to defective transport across the placenta and diminished fetal uptake by using stable isotope technology.

A series of women with appropriate for gestational age (AGA) or SGA fetuses undergoing elective Caesarean section (CS) were infused with stable isotope labelled D-, L-leucine and glycine and mannitol (a non-metabolizable extracellular marker used here for comparative determination of the diffusion rate of D-leucine) to achieve steady state conditions. Maternal blood was sampled every 30 minutes until the baby was delivered. At CS, blood was collected every 30 minutes until the baby was delivered and umbilical vein blood flow was measured using Transit-time flowmetry. Blood was then collected from the umbilical vessels after which the baby was given 13C labelled mannitol via the umbilical vein. At the age of one hour, 2 ml of blood was collected from a vein on the dorsum of the baby’s hand. Plasma analysis of amino acids and their tracers was processed using gas chromatography mass spectrometry.

The results of this thesis include the following:-

• 54 fetuses were studied, of which 33 were appropriate for gestational age (AGA) and 21 SGA. The mean birthweight of the AGA fetuses (3227 ± 471g) was significantly higher than that of SGA fetuses (2361 ± 245g).

• The overall mean umbilical vein blood flow was 86 ± 24 ml. kg.−1 min.−1. Blood flow was significantly lower in SGA fetuses (66 ± 23 ml. kg.−1 min.−1) compared with AGA fetuses (90 ± 18 ml. kg.−1 min.−1).

• Mannitol clearance across the placenta decreased as maternal mannitol concentration increased indicating saturation of a transfer process. Mannitol transfer across the placenta was therefore thought to be carrier mediated rather than passive as had been previously thought and implicitly cannot be used as a good extracellular marker. The mean clearance of mannitol in SGA pregnancies (5.6 ± 2.1 ml. min.−1) was significantly lower than in AGA pregnancies (12.8 ± 1.6 ml. kg.−1 min.−1).

• L-leucine transport across the placenta was significantly lower in the SGA fetuses. Total fetal uptake in AGA fetuses was four times that in the SGA. D-leucine concentration in the umbilical vein was always higher than in the maternal arterIALIZED blood suggesting an active transport mechanism. The fractional extraction ratio of D-leucine was similar to that of L-leucine suggesting that D- and L-Leucine may share a common transporter.

• Glycine transport and fetal uptake were significantly lower in the SGA group. In addition, neonatal glycine levels were significantly lower in the AGA group.

• Fetal oxygen consumption measured as a proxy for fetal metabolic rate showed a high oxygen consumption rate in AGA fetuses (8.4 ± 2.1 versus 6.4 ± 2.6 ml. kg.−1 min.−1). The rate of oxygen consumption increased with birthweight in an exponential relation (VO2 (ml.kg.−1 min.−1) = 4.17Me0.55(kg)). The transport of D- and L-leucine and glycine across the placenta is defective in SGA pregnancies. In addition, fetal uptake of these amino acids is significantly reduced. D-leucine is actively transported across the placenta and the same transporter may be involved in carrying D- and L-leucine. Glycine metabolism in the SGA neonates is significantly slower than that of AGA neonates.
Acknowledgements

I am very grateful to all who have assisted in one way or the other to the successful completion of this work.

I would like to acknowledge the contribution of all the women who participated in this study. They endured the ordeal of intravenous infusions, warm and flushed hands and arms and frequent blood sampling while patiently awaiting the delivery of their babies. Without their support and that of their partners, there would have been no studies. As for their babies, they were remarkably submissive considering what I had to do to them one hour into this world!

I am particularly indebted to Professor Michael John Rennie, Symers Professor of Physiology, University of Dundee for the original concept and useful suggestions on how to interpret the results. The staff at the laboratory in the Department of Anatomy and Physiology, University of Dundee especially Dr. Kenneth Smith and Mr. Shaun Downie were very helpful during the difficult and sometimes extremely frustrating times I had while processing the samples.

A big thank you to all the staff at the Leicester Royal Infirmary Hospital Maternity for the support they gave me. They ensured that all women undergoing elective caesarean sections during the study period were directed to me and their encouraging words to the women were extremely
reassuring and made recruitment less difficult. I would like to express my gratitude to Sister Jan Price of the Maternity Theatre. She always ensured that the equipment in the theatre and the specimen bottles including labels were ready and properly arranged for me.

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I am very indebted to the Pharmacy department of the Leicester Royal Infirmary Hospital. After the initial prolonged and very time consuming experience with preparing the amino acids and mannitol, Janice Lott and Christine Clarke took charge and ensured that I had the amino acids and mannitol prepared on demand. Not only were these two very accommodating but the entire staff of the Intravenous Additive Unit of the whole department was extremely friendly. Thank you all.

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to do the research and was always there for me. He did not only ensure that I had everything that I required for the study but that I had easy journeys to Dundee and while there, always had somewhere to stay. He encouraged me when I was despondent and gave me reasons to carry on when the laboratory experiments were unsuccessful. I cannot find words to express how grateful I am to him. Thank you very much for everything and for reading this Thesis.

Finally, I would like to thank my family and Emma for their support and encouragement during the study. All of this would not have been possible without the guidance of God. Thank you Lord for your kindness in preserving my sanity during this study.

This study was funded by a grant from Action Research to who I am very grateful.
Declaration

Some of the data contained in this thesis have been published in abstract form. Some have been presented at meetings of European Society for Perinatal Medicine, European Society for Parenteral and Enteral Nutrition, The Physiological Society, Joint British and Irish Perinatal Society, Blair Bell Research Society Meeting and the Joint Blair Bell and British Paediatric Society Meeting. Copies of the abstracts published in the book of abstracts from these meeting are enclosed.

In addition a full length paper on umbilical vein blood flow has been published in the British Journal of Obstetrics & Gynaecology, while three other papers have been written and are being submitted to various peer reviewed journals.

This study was approved by the Ethics Committee of the Leicester Health Authority.

I declare that the work presented in this thesis is my own and has not been submitted nor will it be submitted elsewhere for any other degree.

Signed [Signature]

Date. 11. 2 - 97
Critique of the Thesis

Although the results presented in this Thesis appear under two distinct groups the following limitations need to be recognised when interpreting them.

1. The women studied were predominantly between 37 and 39 completed weeks of gestation; a few of them were studied at various gestational ages from as early as 32 weeks gestation. The implications of this variation in gestational ages at which the studies were performed is that the various physiological processes involved with amino acid and mannitol transport may indeed vary by gestation. This is evidenced by the changes in blood flow which have been demonstrated in various studies. Correction for this factor was not possible as the numbers involved were small.

2. The groups studied were divided simply into small for gestational age and appropriate for gestational age based on birth weight for gestational age alone. These groups are unlikely to be as distinct as stated. It is well recognised that being small or of appropriate size at birth is not necessary indicative or exclusive of pathology respectively. It is therefore conceivable that some of the fetuses studied might have been small for gestational age but not pathologically small (i.e. not growth retarded) while some of those that were considered as appropriate for gestational age might have been pathological growth retarded. Since the various processes studied are thought to be different in pathological and non-pathological pregnancies, the interpretation of the results must be made with caution.

3. Some of the pregnancies studied in both groups were complicated by various pathological processes such as pre-eclampsia and diabetes mellitus. The consequences of these processes on the physiological processes studied is unclear. If these processes are altered in these complicated pregnancies, then they may in fact affect the general interpretation of the results. The numbers in the Thesis are, however, small and therefore it was difficult to correct for these disorders.
4. Since this is a preliminary exploration of this topic, the results should provide a basis for planning further studies. Since the results have demonstrated some differences between the two broad groups, subsequent studies will need to take account of gestational age and pathology of pregnancy.
CHAPTER 1

INTRODUCTION

1.1 INTRAUTERINE FETAL GROWTH

1.2 THE HUMAN PLACENTA

1.3 AMINO ACID TRANSFER ACROSS THE PLACENTA
1.0 Introduction

Intrauterine growth retardation (IUGR) is the second most common cause of perinatal morbidity and mortality in the UK. Various factors have been identified as being responsible for IUGR, but the aetiology is unknown in most cases. Although poor intrauterine growth can be a reflection of the fetal genotype, morbidity and mortality associated with IUGR is thought to occur either because of limited substrate availability due to reduction in utero-placental perfusion, low maternal substrate concentration or reduced placental transfer. For normal fetal growth to occur, the whole range of nutrients such as oxygen, amino acids, carbohydrates, lipids, vitamins and minerals must be provided in adequate amounts. An effective delivery of these nutrients depends on blood flow to the placenta and fetus and the transfer of the nutrients across the placenta.

Several investigators (Ghadimi & Pecora, 1964; Young & Prenton, 1969; Economides et al. 1989 and Cetin et al. 1989, 1990) have demonstrated that the plasma concentration of total and some amino acids notably that of valine, glycine, leucine, isoleucine and lysine is lower in growth retarded fetuses than in appropriate for gestational age fetuses. This difference could be due to either an increase in fetoplacental consumption of these amino acids, reduced perfusion of the placenta or reduced placenta amino acid transporter numbers and function. There is some evidence for reduced placental transportation in that the transport of the non-metabolizable artificial amino acid 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) and methyl amino-isobutyric acid (MeAIB) into vesicles of microvillous membranes has been shown to be reduced in the placentae of small for gestational age pregnancies (Dicke and Henderson, 1989; Mahendra et al. 1991).
This thesis describes in-vivo studies on amino acid transport in human gestation. Two transport systems (System L - transporting the neutral amino acid leucine and System Gly - transporting glycine) were studied. Since the uptake of various nutrients depends on blood flow, umbilical vein blood flow was therefore also compared in small and appropriate for gestational age fetuses. Fetal metabolic rate (oxygen consumption) - an indirect assessment of the rate of utilisation of these nutrients was also measured at caesarean section. In this chapter an overview of intrauterine fetal growth (including the clinical problems of the growth retarded and macrosomic fetus) will be discussed, followed by brief reviews of the human placenta (anatomy, circulation and nutrient transfer mechanisms through it) and, amino acid (including chemistry and physiology) transport.
1.1 INTRAUTERINE FETAL GROWTH

1.1.1 Introduction

1.1.2 Clinical implications of fetal undergrowth (intrauterine growth retardation)

1.1.3 Clinical implications of fetal overgrowth (Macrosomia)

1.1.4 Intrauterine fetal growth - patterns

1.1.5 Early Intrauterine Fetal Growth

1.1.6 Regulation of fetal growth

1.1.7 The utero-placental unit in the regulation of fetal growth
INTRAUTERINE FETAL GROWTH

1.1.1 Introduction
The prenatal growth period is very important to the human's future well-being. Although intrauterine growth is determined by the fetal genome, it is often regulated to a large extent by maternal supply and placental transfer and exchange of nutrients. When there is an imbalance in this intricate system of growth regulation, the result is either fetal undernutrition (intrauterine growth retardation) or overgrowth (fetal macrosomia). These growth abnormalities are associated with complications during pregnancy, labour and the neonatal period (Jones and Robertson, 1984; Creasy and Resnik, 1989) and have recently been linked with the occurrence of diseases in adult life (Barker et al., 1991, 1993, 1995).

1.1.2 Clinical Implications of intrauterine growth retardation:

1.1.2a Definition:
In 1948, the World Health Organisation defined low birth weight babies as those weighing less than 2500 grams (UNO, 1955). Though these babies were initially regarded as preterm, it soon became clear that as many as one third were actually born after 37 completed weeks of gestation. Battaglia (1970) and Yerushalmy (1970), therefore subdivided these babies into three categories:

i) preterm and appropriate for gestational age,

ii) preterm and small for gestational age and

iii) term and small for gestational age.
Clifford (1954) was the first to describe certain morphological features of growth retarded babies especially those born at term and these were later confirmed by Gruenwald (1965) and Naeye (1965).

Though Obstetricians and Neonatologists define intrauterine growth retardation (IUGR) by an arbitrarily birth weight below the 10 centile it is well known that many normally grown fetuses are included in this category and that some babies who are bigger than 2500 grams or over the 10th centile may not have achieved their growth potential and are in fact growth retarded. An alternative definition of below the 3rd, 5th centiles and 2SD below the mean weight for gestational age are more stringent and therefore more likely to include a higher proportion of growth retarded infants. Recently, there has been a tendency to use the term small for gestational age (SGA) rather than the term intrauterine growth retardation to emphasise that being small at birth is not necessary pathological.

1.1.2b Incidence:

The incidence of intrauterine growth retardation varies depending on the population, geographic location and standard curves used as reference and the definition (Creasy and Resnik, 1989). In developed countries, the incidence of babies weighing less than 2500 grams at birth is between 4 and 7 percent (Gruenwald, 1963; Scott and Usher, 1966; Lugo and Cassady, 1971; Galbraith et al., 1979)
1.1.2c Problems of Growth Retarded Babies:

The prenatal, intrapartum and post-natal complications associated with intrauterine growth retardation (IUGR) remain a major cause for concern for the Obstetrician and Neonatologists. It has been estimated that Obstetricians and Neonatologists spend about a third of their time looking after pregnancies and babies complicated by intrauterine growth retardation (Creasy and Resnik, 1989).

Intrauterine growth retarded babies are more likely to be delivered prematurely and have a higher incidence of unexplained stillbirths (Yudkin et al., 1987) and of antepartum and intrapartum fetal hypoxaemia and acidosis (Creasy & Resnik, 1989; Soothill, 1989).

Perinatal Mortality:

Intrauterine growth retardation is one of the most common causes of perinatal mortality in the UK. Butler and Bonham (1963) in the British Perinatal Survey found that in mature or prolonged pregnancies, low birth weight babies (<2500g) had a mortality eight times that for babies weighing more than 2500 grams. The perinatal mortality rate is said to be doubled in those babies 15-25% under the mean birth weight for gestation (Creasy and Resnik, 1989), increases eight fold in those below the 3rd centile and rises to 50% in those who are 40% or more underweight (Scott and Usher, 1966). More recent studies show that the perinatal mortality in infants born after 37 completed weeks but weighing between 1500 and 2500 grams is 30 times that of babies between the 10th and 50th centiles and 70 to 100 times higher in those weighing less than 1500 grams (Williams et al., 1982).
In the neonatal period, the potential problems include:

**Birth asphyxia:**
This is 5-10 times more common in growth retarded babies (Walther and Ramaekers, 1982; Jones and Roberston, 1984). MacDonald et al. (1980) reported incidences of 36.0% and 6.2% in growth retarded and appropriate for gestational age babies respectively born before 36 weeks and 4.1% and 0.36% in those born after 36 weeks.

**Hypoglycaemia:**
This was first recognised by Cornblath et al. (1959) and it is commoner in the asymmetrically growth retarded babies (Walther and Ramaekers, 1982). Hypoglycaemia has been reported to be eight times more common in the growth retarded baby than its appropriately grown counterpart (Neligan et al., 1963).

**Hypothermia:**
This results from lack of subcutaneous fat, increase heat loss from a larger surface area and inadequate non-sweating thermeogenesis (Aherne and Hull, 1966; Hey, 1971; Sinclair, 1970 and Walther and Ramaekers, 1982).

**Haematological Problems:**
The main haematological complication of IUGR babies is polycythaemia (Wirth et al., 1979). Finne (1966) stated that this is as a result of a rise in erythropoietin levels in response to chronic in-utero hypoxia. Polycythaemia may in turn lead to other complications such as central nervous system depression, convulsions, venous thrombosis, respiratory distress, jaundice, hypoglycaemia, hypocalcaemia and heart failure (Jones and Robertson, 1984). Prolonged
prothrombin time and thrombocytopenia have been reported (Peelman and Dvilansky, 1975; Shuper et al., 1983) respectively but these are, however, uncommon (Jones and Robertson, 1984).

**Pulmonary Disease:**
Though meconium aspiration syndrome is not generally considered more common in IUGR neonates (Jones and Robertson, 1984), when it occurs, it tends to be more severe (Creasy and Resnik, 1989). Intrapulmonary haemorrhages are generally more common in IUGR neonates (Fedrick and Butler, 1971; Sly and Drew, 1981) though some units have not reported this difference (Jones and Robertson, 1984). Respiratory distress syndrome secondary to prematurity is not common in IUGR babies. Overwhelming evidence suggest that the incidence is in fact lower than that in appropriate for gestational age neonates (Procianoy et al., 1980). However, polycythaemia may predispose to respiratory distress syndrome.

**Neurodevelopmental Handicap:**
As early as 1862, Little recognised that babies weighing less than 2500 grams were predisposed to neurodevelopmental handicap. Since then, various studies have shown that growth retarded babies are at an increased risk of cerebral palsy (Griffiths and Barrett, 1967; Durkin et al., 1976; Nelson and Broman, 1977), more neurological defects and poorer development (Fitzhardinge et al., 1978) and lower intelligence quotient (Francis-Williams and Davies, 1974; Taylor, 1984). Long term follow up of growth retarded babies has shown that those born prematurely are more frequently handicapped either physically or neurologically (Fitzhardinge and Stevens, 1972; Ounsted and Ounsted, 1973; Ounsted et al.,
1981). Low et al. (1975), however, observed that term growth retarded babies were not necessarily at an increased risk of neurodevelopmental handicap.

**Congenital Malformations:**
The incidence of minor and major congenital malformations in IUGR fetuses varies between 6 and 30% (van den Berg and Yerushalmy, 1966; Fortune and Kitchen, 1977; Andreasson et al., 1981; Ounsted et al., 1981). These malformations are structural, chromosomal or secondary to intrauterine infections such as rubella, syphilis and cytomegalovirus.

**Morbidity and mortality in adult life:**
Recently, it has been shown that prenatal events influence the incidence of various disease conditions in adult life. Low birthweight is associated with an increase in the incidence of cardiovascular diseases such as hypertension and heart attacks in adult life (Barker et al., 1989 and 1993; Law et al., 1993), chronic lung diseases (Barker et al., 1991) and diabetes mellitus (Barker et al., 1993). These findings have also been demonstrated in experimental animals subjected to intrauterine malnutrition (Langley and Jackson, 1994).

### 1.1.3 Clinical Implications of Fetal Overgrowth (Macrosomia).

#### 1.1.3a Definition and incidence
The definition of fetal overgrowth or macrosomia varies from birthweight over 4000 grams after 37 completed weeks of gestation to birth weight over 4500 grams after 37 weeks. For the former figure, the incidence is about 7-8% and for the latter, it is 1-2% (Brundell, 1989). Macrosomia may occur where there is no known cause (for example constitutional) or if the baby is abnormal as in
Beckwith-Wiedeman's syndrome, or has diabetes or is born to a mother with diabetes mellitus. Factors such as race, birth order, maternal weight and height and social class all affect the incidence of fetal macrosomia to an extent.

1.1.3b Problems of macrosomic babies

Macrosomic fetuses present problems at delivery and in the neonatal period. These problems include those of shoulder dystocia and higher operative delivery rates while the neonatal problems tend to depend on the aetiology of the macrosomia (Neiger, 1992). The neonate of the diabetic mother for example is at an increased risk of hypoglycaemia, hypocalcaemia, hypomagnesaemia, jaundice, polycythaemia, respiratory distress syndrome and congenital malformations (Chervenak, 1992).

1.1.4 Early Intrauterine Fetal Growth Patterns:

Fetal growth and its regulation remain poorly understood. From the very beginning of life, the forces responsible for selecting the one sperm out of millions which fertilizes the ovum are unknown. However, by the time of implantation, the blastocyst consists of 150 cells. After implantation, the outer layer of the blastocyst undergoes a series of changes which culminate in the formation of the placenta while the inner layer undergoes differentiation to form the various organs of the fetus. This period from the second to the eight week referred to as the period of embryogenesis, is hazardous as 10% of all fertilized ova do not reach it and 50% of those that do abort spontaneously, often without the mother being aware she was pregnant. In the embryonic period, the velocity of growth is slow. This is because, differentiation of the originally homogeneous
cell mass into various regions and organs of the body, such as the head and arms, is the predominant event and occurs as histogenesis (the differentiation of cells into specialized tissues such as muscle and nerves). Up till the 26th postmenstrual week, the increasing fetal weight is due mainly to protein accumulation as the cells of the body are formed. From then on, fat begins to accumulate and increases from 30 grams at 30 weeks to 430 grams at 40 weeks (Southgate and Hey, 1976). At term, the body composition of the fetus is 69% water, 14% protein, 13% fat and the remaining 4% is made up of bone minerals (Widdowson et al. 1988).

1.1.5 Intrauterine Fetal Growth

Francois Mauriceau (a French Obstetrician) was perhaps the first to record birth weights. Jonnes Roedever (a German Obstetrician) was the first to publish an accurate report on birth weights (1753) in Gottingen. He found the average male infant's weight to be 3050 grams and the female's to be 2800 grams. In 1946, McBurney presented cases of undernourished full term infants - thus introducing the concept of intrauterine growth retardation.

Fetal weight gain patterns have been derived from cross-sectional studies and extrapolations from growth curves. In the early part of pregnancy, weight gain is difficult to estimate but various studies in the second and third trimester conclude that the average fetal weight at 24 weeks is 759 grams, 1140 at 28 weeks, 2319 grams at 34 weeks and 3753 grams at 40 weeks (Wilcox et al. 1993). The average weight gain between 24 and 28 weeks is 10 - 16 grams per day, 28 - 36 grams per day between 32 and 37 weeks (Sterky et al. 1956; Thompson et al. 1965; Wilcox et al. 1993) and 20 - 26 grams per day between 37 and 40 weeks.
(Thompson et al. 1965; Sterky 1970; Wilcox et al. 1993) in the appropriate for gestational age fetuses. In small for gestational age fetuses, weight gain is 19 grams per day between 32 and 37 weeks and ceases after 37 weeks while large for gestational age fetuses gain 39 grams between 32 and 40 weeks and 42 grams per day after 42 weeks (Sterky 1970). Table 1.1 shows the average fetal weight gain at different stages during human gestation.

**Table 1.1:** Estimated average weight gain (g/kg/day) during different stages of pregnancy (From Owen, Unpublished data)

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>3rd Centile</th>
<th>10th Centile</th>
<th>50th Centile</th>
<th>90 Centile</th>
<th>97th Centile</th>
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<td>23.8</td>
<td>23.9</td>
<td>26.1</td>
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</table>
1.1.6 Regulation of Fetal Growth:

The growth of the fetus is determined by the genome constrained by imperfect maternal supply and placental exchange of nutrients (Gluckman & Liggins, 1986). The rate of fetal growth therefore represents the balance between constraining and stimulating factors acting on the genetically programmed drive to growth. In the first half of pregnancy, genetic control is dominant and gives rise to relatively narrow limits of variability of patterns of fetal growth; in the second half of pregnancy, constraints and stimuli become increasingly important and give rise to a greater variability of growth and maturational milestones (Gluckman & Liggins, 1986).

1.1.6a Genetic control of fetal growth:

The way in which genetic information contained in a fertilized egg guides cell multiplication and differentiation that results in the attainment of the mature human form is unclear. A tightly programmed sequence of gene activation and suppression is, however, necessary for the development to proceed in an organised manner that allows particular developmental events to occur at precise gestational ages (Gluckman & Liggins, 1986). This programme is contained within the genome. Single gene defects such as cystic fibrosis and chromosomal abnormalities such as trisomies also influence birthweight (Robson, 1978). Reduced birthweight in trisomies is due to a reduction in cell multiplication rate (Robson, 1978).
The contribution of genetic influence to birth weight is thought to be about 38 %, of which 20 % is from the mother, 16 % from the fetus and 2 % from the fetal sex. The environment contributes 62 % to the growth of the fetus (Polani, 1974).

1.1.6b Embryonic genotype:

The embryonic genotype as a whole is probably instrumental in governing embryonic/fetal growth until the maternal supply becomes limiting. Sex has an important influence on size and growth; males are generally larger than females. This might be due to enhanced growth of the male fetus ascribed to the anabolic effects of male gonadal hormones and larger placentae resulting from a greater antigenetic difference between the mother and her male fetus. In mice, it does appear that differences in growth are present in the very early stages when hormonal and placental influences are minimal, suggesting that the genotype on its own has an influence on size.

1.1.6c Maternal factors affecting fetal growth:

Superimposed on the genetic control of fetal growth is the maternal influence termed "maternal constraint". Walton and Hammond (1938) demonstrated this maternal constraint vividly when they crossed Shire horses with Shetland ponies. The weights of foals born to the Shires were similar to those of pure Shires while those born to ponies were similar to those of pure Shetland pony breeds. Though this experiment showed that maternal factors override the part of fetal genetic make-up, it did not clearly distinguish between those maternal influences expressed through genes of the fetus and those expressed through the fetal environment. Smidt et al. (1967), by transplanting fertilized eggs eliminated the
genetic component and showed that maternal factors were overriding. Eggs from normal sized pigs transplanted into dwarfed sows produced dwarfed piglets while eggs from dwarfed pigs transplanted into normal sows produced normal sized piglets. Venge (1950) and Hunter (1956) made similar observations in sheep and rabbits respectively. In man, there is indirect evidence for maternal constraint on fetal growth. There is a better correlation between birthweights of half sibs with a common mother rather than those with a common father (Morton, 1955).

1.1.6d Growth factors in the regulation of fetal growth:

Little is known about how growth and differentiation is regulated in the early human embryo. In particular, little has been revealed about the mechanisms by which polypeptide growth factors and hormones influence important events during human embryogenesis. There is, however, accumulating evidence that specific growth factors/hormones as well as their appropriate receptors are expressed in early embryonic development. This evidence strongly suggest that the fetus is dependent on polypeptide growth factors and hormones for growth and differentiation. These factors can in principle be supplied to the fetus in three ways. Firstly, they can be produced by the mother and then transported to the fetus through the placenta. Secondly, growth factors may be produced by specialized extraembryonic tissues such as the yolk sac and thereafter are released into the fetal circulation (Shi and Heath, 1984; Shi et al. 1985) and thirdly growth factors can be produced and act locally within the fetal tissues (Engstrom & Heath, 1988). Growth factors and hormones which may influence fetal growth include epidermal growth factor (EGF) (Cohen & Taylor, 1975; Barka et al. 1979; Goldin & Opperman, 1980; Sundell et al. 1979; Messmer et al. 1982; Adamson &

1.1.6e The utero-placental unit in the regulation of fetal growth:

While the fetal genome and the maternal genetic constitution are very important in the control of fetal growth in the early gestational period (contributing up to 38% of the control of fetal growth), maternal constraint manifested mainly as the environmental influence, plays an important role in the regulation of fetal growth in the second half of pregnancy. This is achieved via the influence of the utero-placental unit.

Satisfactory fetal growth and development will depend on a utero-placental unit that is functioning properly. This unit provides the rapidly growing fetus with various essential nutrients including amino acids, fatty acids and lipids, glucose and other carbohydrates, gases etc., while at the same time acting as an excretory organ for the end products of fetal metabolism. The utero-placental unit has also been shown to be an active synthetic source of some fetal nutrients. An efficient utero-placental unit must have an adequate blood supply via the uterine vessels, a normal placenta architecture and a normal blood flow from the utero-placental unit to the baby. These factors and the concentrations of the various nutrients in the maternal plasma to and from the unit to a large extend determine the rate at which the growing fetus acquires various nutrients from the utero-placental unit.
It is well recognised that in some growth retarded pregnancies, the functions of the utero-placental unit are impaired. In sheep, reduction in uterine blood flow to below 50% is associated with a significant reduction in the metabolic rate of the utero-placental unit and therefore that of the lamb and a similar step-wise reduction in umbilical vein blood flow in chronic sheep preparations has shown that fetal metabolic rate is reduced (Emmanouildes et al. 1968). In humans, studies performed on umbilical vein blood samples obtained at mid and late gestation by cordocentesis and at delivery and from the maternal vessels have shown marked differences in fetal oxyhaemoglobin, some essential and non-essential amino acids, fatty acids and glucose levels in normal and growth retarded fetuses and babies (Gluckman & Liggins, 1986) confirming the role of the utero-placental unit in fetal growth.

Teasdale and Jean-Jacques (1985) have also examined the morphometry of placentae from normal pregnancies and those complicated by intrauterine growth retardation and have shown that in the growth retarded pregnancies, the placental morphometry is abnormal.

While very extensive studies have been carried out in animals on the exchange rates and transport functions of the placenta (in-vivo and in-vitro), and blood flow (both uterine and umbilical vein) ethical considerations and technical problems have restricted such studies in human pregnancy. Data on the transport (especially of amino acids) and gaseous exchange functions of the human utero-placental unit and fetal metabolism are therefore lacking. In fact values often quoted for humans have been obtained by deductions from animal
studies especially those from the sheep despite well recognised differences between human and sheep placentae.
The main objective of this research was therefore to study some aspects of the utero-placental unit and fetal metabolism. In order to do this, it was important to study some aspects of materno-placento-fetal transfer first. This was achieved by:

1. measuring umbilical vein blood flow by a novel technique hitherto used in vascular and veterinary surgery,

2. determining the transfer of a non-metabolizable compound (mannitol across the placenta,

3. determining the nature of observed altered feto-maternal amino acid gradients (especially for some essential amino acids and glycine),

4. determining the nature of D-amino acid transport across the placenta of growth retarded and appropriate for gestational age pregnancies; and

5. determining the fetal metabolic rate indirectly by measuring the fetal oxygen consumption,
1.2 THE HUMAN PLACENTA

1.2.1 Introduction

1.2.2 Development of the fetal placenta

1.2.3 Development of the maternal placenta

1.2.4 Anatomy of the fetal placenta

1.2.5 The fetal circulation through the placenta

1.2.6 The maternal uteroplacental circulatory system

1.2.7 Transfer across the placenta
1.2.1 Introduction

The human placenta is a villous, haemochorial structure. It is of critical importance in materno-fetal transfer, has a complex synthetic capacity and plays a fundamental role in the immunological acceptance of the fetal allograft. The placenta is unique in its range of functional activities, its ability to flourish in an immunologically alien environment and its vascular parasitism (because it depends upon maternal blood, for its oxygenation and nutrition). There are two main components to the human placenta - the fetal component which is usually expelled from the uterus and the maternal component which comprises the placental bed and the uteroplacental vessels. Both components are of pivotal important in fetal nutrition and therefore growth.

To be able to study the role of the placental unit in the transport of various nutrients (especially gases and amino acids) and fetal metabolism, it is important to understand the development, anatomy, circulation and the transport mechanisms of this organ. This chapter therefore reviews these aspects of the placenta.

1.2.2 Development of the fetal placenta:

The fertilized ovum enters the uterine cavity as a morula. It is rapidly converted into a blastocyst with the formation of a fluid filled cavity. The cells of the blastocysts differentiate into an inner cell mass (located mainly to one pole of the blastocysts) and an outer primary trophoblastic cell mass. The former differentiates into various fetal organs while the latter differentiates to become
the placenta. Cells from the trophoblastic mass infiltrate between those of the endometrial stroma (decidua). At about the 10th or 11th postovulatory day, this invasion is complete (implantation) and the blastocysts is now surrounded by decidua. At this stage, the trophoblast which forms a peripheral circumferential plaque, rapidly differentiates into an inner layer of large mononuclear cytotrophoblastic cells with defined limiting membranes and an outer layer of multinucleated syncytiotrophoblast. The latter layer is derived from the former and is formed by the breakdown of the limiting membrane of the cytotrophoblastic cells.

By the 10 - 13th post ovulatory days, intercommunicating lacunae appear in the rapidly enlarging trophoblastic cell mass and then soon become confluent to form the precursors of the intervillous spaces. As maternal vessels are progressively eroded, these spaces become filled with maternal blood. At about day 14th to 21, trabecular columns of syncytiophoblast incompletely separate the lacunae from each other. The columns soon come to possess a central cellular core that is produced by the proliferation of the cytotrophoblastic cells at the chorionic base. The "placenta" (at this stage ) is a labryinthine rather than villous structure. The trabeculae are known as Primary Villous Stems (Boyd and Hamilton, 1970).

Continuous growth of the cytotrophoblasts leads to its distal extension into the region of decidual attachment. At the same time, a mesenchymal core appears within the villous stems, formed by a distal extension of the extraembryonic mesenchyme. The Villous Stems then become vascularized - the vessels developing from mesenchyme within the core and establishing, in due course,
functional continuity with other vessels differentiating in the body stalk and inner chorionic mesenchyme.

The distal part of the villous stem at this stage is formed almost entirely by cytotrophoblastic cells which form columns anchored to the decidual basal plate. The cells in these cytotrophoblastic cell columns proliferate and spread laterally to form a continuous cytotrophoblastic shell which splits the syncytiotrophoblast into two layers, the definitive syncytium on the fetal side of the shell and the peripheral syncytium on the maternal side. The definitive syncytium persists as the lining of the intervillous space, but the peripheral syncytium eventually degenerates and is replaced by fibrinoid material (Nitabuch's layer). Sprouts from the primary villous stems spread into the intervillous space. The primary stem villi grow and divide to form secondary and tertiary stem villi, the latter eventually into the terminal villous tree. By this stage, the placenta is a villous and vascular organ. Between now (21st postovulatory day) and the fourth month, the placenta undergoes considerable growth and remodelling. The villi orientated toward the uterine cavity degenerate to form the chorion laeve while the thin layer of decidua (decidua capsularis) covering this area gradually disappears allowing the chorion to eventually come in contact with the decidua parietalis on the opposite wall. The villi on the side of the chorion orientated towards the decidual plate proliferate and progressively aborize to form the chorion frondosum which develops into the definitive fetal placenta.

The placenta septa which protrude into the intervillous spaces from the basal plate and divide the maternal surface of the placenta into 15-20 lobes appear during the third month. They are simple folds of basal plate formed partly as a
result of regional variability in placental growth and partly by the pulling up of
the basal plate by the anchoring columns which have a poor growth rate. By the
end of the fourth month, the fetal placenta has achieved its definitive form and
undergoes no further anatomical modification. Growth, however, continues
until term and is due mainly to continuing branching of the villous tree and
formation of fresh villi.

1.2.3 Development of the Maternal Placenta

During the early weeks of pregnancy, cytotrophoblastic cells stream from the tip
of the anchoring villi, penetrate the trophoblastic shell and extensively colonize
the decidua and adjacent myometrium of the placental bed. These cells are
known as the interstitial extravillous cytotrophoblast. Trophoblastic cells, in
addition, also stream into the lumens of the intradecidual portions of the spiral
arteries of the placental bed where they form intraluminal plugs and are known
as the intravascular extravillous cytotrophoblast. These endovascular
trophoblastic cells destroy and replace the endothelium of the maternal vessels
and then invade the media with resulting destruction of the medial elastic and
muscular tissue (Roberston et al., 1975). The arterial wall is then replaced by
fibrinoid material, which is derived partly from fibrin in the maternal blood and
partly from protein secreted by the invading trophoblastic cells. This process
(referred to as the first wave of trophoblastic invasion) is completed by the end of
the first trimester, at which time these "physiological" changes within the spiral
arteries of the placental bed extend to the myometrio-decidual junction.

Between the 14th and 16th weeks of gestation, there is a resurgence of
endovascular trophoblastic migration, with a second wave of cells moving down
this time, into the intramyometrial segments of the spiral arteries. This invasion (second wave) extends as far as the origin of the spiral arteries from the radial arteries. Within the intramyometrial portion of the spiral arteries, there is endothelial replacement, invasion and destruction of the medial musculoelastic tissue and fibrinoid changes in the vessel wall. The end result is a change from thick-walled, muscular spiral arteries to flaccid, sac-like uteroplacental vessels, which can passively dilate in order to accommodate greatly augmented blood flow through its vascular system that is required as pregnancy progresses.

In the absence of the activities of the extravillous trophoblastic cells, the placenta fails to establish its own low-pressure, high conductance vascular system, leading to inadequate maternal blood flow to itself with the result that fetal oxygenation and nutrient supply is poor.

The factors controlling and limiting intravascular invasion by extravillous trophoblast are unknown, but the crucial importance of the process is shown by the finding that in women destined to develop pre-eclampsia in the later stages of pregnancy, there is a partial/complete failure of placentation, resulting in a markedly restricted blood flow to the placenta. Khong et al., (1986) showed that in pre-eclamptic pregnancies, only a proportion of the spiral arteries in the placental bed (compared to all the spiral arteries, in uncomplicated pregnancies) are invaded by trophoblast and that even in these vessels, the "physiological" changes occurred only in the intradecidual segments, there being a complete failure of the second wave with endovascular trophoblast failing to advance into the intramyometrial portion of these vessels. The consequences of such failure is
either pre-eclampsia or intrauterine growth retardation in normotensive pregnancies (Robertson et al., 1981) due to reduced maternal supply of oxygen and nutrients to the developing fetus.

The exact function of the interstitial extravillous trophoblastic cells is obscure. They tend to aggregate around the spiral arteries and it has been suggested that they prime these vessels to allow them to react to their eventual invasion by endovascular trophoblast (Pijnenborg et al., 1983). The exact mode of action on these vessels is, however, unclear.

1.2.4 Anatomy of the fetal placenta

The fetal placenta is made up of a number of subunits generally known as lobules. The primary stem villi divide just below the chorial plate into a number of secondary stem villi which, after running for a short distance parallel to the chorionic plate, subdivide into a series of tertiary stem villi which sweep down through the intervillous space to anchor on to the basal plate; during their course through the intervillous space, they give off multiple branches which ramify into the terminal villous network. As the tertiary stem villi pass down towards the basal plate, they are arranged in a circular fashion around the periphery of an empty cylindrical space: the lobule forms a hollow globule with the bulk of the terminal villi being mainly in the outer shell of this globular structure and the centre of the lobule being relatively empty and free of villi. The lobules are separated from each other by interlobular areas which are in continuity with the subchorial space.
From each primary stem villous is derived a cotyledon - a term often confused with lobes seen on the maternal surface of the fetal placenta or lobules as described above. It is restricted to a functional unit of the placental villous tree derived from a single primary stem villus and therefore may contain a differing number of lobules depending on the varying number of secondary and tertiary stem villi. Fox (1981) advocated that the human placenta is not really a cotyledonary structure and therefore the word cotyledon should be abandoned.

1.2.5 The fetal circulation through the placenta

Fetal blood passes to the placenta through the two umbilical arteries which spiral around the umbilical vein. Shortly before reaching the placenta the two arteries are connected by one or more anastomotic vessels, and may even fuse into a single trunk which subsequently divides into two rami. On reaching the placenta, the arteries run in the chorion; they are usually of equal size and each supplies half of the organ. As the arteries run across the chorion, they branch repeatedly, a proportion of the branches at each division entering the placental substance to run in the primary stem villi. These cotyledonary arteries soon divide into secondary stem arteries which in turn aborize into tertiary stem arteries. The latter run through the intervillous space within the tertiary stem villi giving off, as they do throughout their course, many villous branches which eventually subdivide into a villous capillary system. The terminal villi are vascularized only by capillary vessels and these are arranged in such a way that three to five terminal villi are supplied by the same multiple coiled capillary loop.
The fetal blood flow through the placenta is about 500ml/min. The main propelling force is the fetal heart, but it is possible that there is also a peripheral villous pulse: smooth muscle fibres are present in the stem and anchoring villi, and contraction of these fibres may help to pump blood from the placenta to the fetus. It has been demonstrated that the fetal vessels in the placenta are ensheathed in myofibroblastic cells (Adamson and Myers, 1975) and contraction of these cells could be an important factor in establishing the villous pulse.

1.2.6 The maternal uteroplacental circulatory system

Maternal blood enters the intervillous space via arterial inlets in the basal plate and is then driven by the head of maternal pressure towards the chorionic plate as a funnel-shaped stream. The driving head of maternal pressure is gradually dissipated, a process aided by the baffling effect of the villi, and lateral dispersion of the blood occurs. This forces the blood already in the intervillous space out through basally sited, wide venous outlets into the endometrial venous network (Ramsey and Donner, 1980).

The physiological basis for the circulatory system is a series of pressure differentials, the pressure in the maternal arterioles being higher than the mean intervillous space pressure, which in turn exceeds that in the maternal veins during myometrial diastole. This entire system is, however, a low pressure one, for whereas in most organs there is a progressive decrease in the diameter of the arteries as they approach their target tissues, the reverse is true for the placenta, the uteroplacental vessels increasing in diameter as they near the intervillous space. There is therefore a considerable drop in pressure from the proximal to the distal portion of these vessels, and the full arterial pressure is not transmitted to
the intervillous space. The placenta itself offers little flow resistance to maternal blood and has a high vascular conductance; there is thus very little fall in pressure across the intervillous space. Despite the fact that the pressure difference between arterial and venous sides of the intervillous space is small, it is apparently sufficient to drive arterial blood towards the chorionic plate, to stop the stream "shorcutting" into adjacent venous outlets and to prevent mixing of neighbouring arterial inflows.

The main factor that controls the adequacy or otherwise of maternal blood flow to the placenta is the extent and degree of conversion of the spiral arteries of the placental bed into uteroplacental vessels by the extravillous trophoblast. Doppler studies have shown that during the early stages of pregnancy, there is a progressive decrease in the peripheral resistance within the uteroplacental vasculature which reaches a plateau between the 22nd and 24th week of gestation i.e at the time when conversion of the spiral arteries to uteroplacental vessels is complete (McParland and Pearce, 1988).

Changes in the perfusion (and pressure) in the radial arteries will not, unless very marked, significantly influence uteroplacental blood flow, largely because there is normally a considerable drop in pressure from the radial arteries to the uteroplacental vessels. Thus the relatively minor changes in arterial pressure associated with maternal anaesthesia or sedation have no perceptible influence on uteroplacental blood; conversely, an increase in maternal blood pressure is unlikely to result in a significant increase in placental perfusion. Vasoconstrictive agents such as nicotine can reduce maternal uteroplacental blood flow, though it should be noted that the uteroplacental vessels themselves are unable to constrict
because of the destruction of their muscular and elastic component by invading trophoblast.

It is also unlikely that the vascular resistance within the intervillous space can be significantly altered by any factor other than uterine contractions. It has often been argued that villous swelling, because of oedema, could reduce the size of the intervillous space and increase resistance within the space. This is, however, a purely theoretical concept which has not received support from clinicopathological studies (Shen-Swarz et al., 1989).

During myometrial contractions the afferent blood flow through the intervillous space may be markedly reduced or even cease. This is probably due to compression and occlusion of the veins draining the intervillous space, but ultrasonic studies have shown that during a myometrial contraction the intervillous space distends and the fetus is not severely deprived of an oxygen supply during myometrial systole.

1.2.7 Placental transfer of nutrients

Although early investigators suggested that the placenta was simply a passive semipermeable sieve, it is now regarded as one organ in the body with many mechanisms of transfer operating simultaneously (Longo, 1972). Through these mechanisms it functions as the fetal kidneys, lungs, liver, gastrointestinal tract and as an endocrine organ. While gases may diffuse between the maternal and fetal circulations, carbohydrates are exchanged by facilitated diffusion and amino acids and vitamins by active transport. Other substances such as globulins
traverse the placenta by the process of pinocytosis and water by bulk flow. These various transfer mechanisms will now be discussed.

The nutrients present in the maternal blood reach the fetal blood stream after crossing the syncytiotrophoblast, and the cytotrophoblast, the connective tissue within the villus and the capillary endothelial cells. The crossing of this anatomical barrier is one of many factors that influence the rate of maternal-fetal transfer. Physiological limitations or pathological changes may affect the rate of exchange or metabolism of these substances; the end result being alteration in fetal growth. Such factors include utero-placental blood flow, the size and chemical characteristics of the substance to be transported, the metabolic activity of the placenta and the fetal circulation.

Studies in other mammals and a rather limited number in humans, indicate that the major mechanisms for maternal-fetal transfer of nutrients are:

1. Simple (passive) diffusion
2. Facilitated Diffusion
3. Active Transport
4. Endocytosis
5. Bulk Flow or Ultrafiltration

1.2.7a Simple Diffusion:

Simple diffusion is the movement of a molecular species across a membrane by random thermal motion from an area of high concentration to one of low concentration. It is a passive process involving no energy consumption or work
by membrane. Simple diffusion usually takes place because of chemical gradients. The net quantity of molecules transferred by simple diffusion is directly proportional to the concentration and/or electrochemical difference across the membrane; hence, simple diffusion which is downhill continues until uniform concentration of electrochemical equilibrium is established. Simple diffusion is also influenced by certain characteristics of the membrane such as area, diffusivity and thickness. The thickness of the placental barrier decreases progressively during gestation, and is likely to increase the rate of transfer of certain nutrients. However, there is evidence that the different thickness of the placental membrane present in different mammalian species does not determine proportional differences in the rate of transfer of freely diffusable substances. Therefore, considerations of facility of transfer based on pure anatomical features are not necessarily valid.

The rate of diffusion across the membranes is also affected by several physiochemical factors such as molecular size, electrical charge and lipid solubility. In general, substances of smaller molecular size diffuse more rapidly than larger molecules; substances such as Na⁺, K⁺, Cl⁻ are placental membrane permeable, while others such as bicarbonate ions are less permeable (Dancis and Schneider, 1975). Lipid-soluble substances are likely to diffuse through the entire cell membrane in accordance with its lipid nature - and rapidly approaches equilibrium between maternal and fetal circulations while lipid-insoluble substances are likely to diffuse more slowly through membrane pores or organ channels between cells.

Examples of substances that are transferred across the placenta by simple diffusion include gases (oxygen, carbon dioxide, carbon monoxide and inert
gases) and lipids such as free fatty acids (Knobil et al, 1959; Van Duyne et al, 1962).

1.2.7b Facilitated Diffusion:

This mechanism is similar to simple diffusion in that it requires the presence of either concentration or electrochemical gradients across the membrane and does not require energy. However, it differs from simple diffusion in that the rate of transfer is much faster, becomes constant at high concentrations, and can be interfered with by the presence of a different molecule with a similar spatial configuration. These characteristics indicate the presence of certain "transfer sites" or "receptor-carriers" that can react or combine with the molecule being transferred. The molecular mechanisms of this form of placental transfer are largely unknown. Glucose and other carbohydrates are examples of substances transferred by this mechanism (Dancis and Scheider, 1975).

1.2.7c Active Transport:

In contrast with the two previous mechanisms, active transport can proceed against both concentration and electrochemical gradients. Similar to facilitated diffusion, active transport can be interfered with by molecules with similar configuration, and it becomes constant at high concentrations; thus, it also requires the presence of a "receptor-carrier". The main characteristic of active transport, however, is its absolute requirement for energy provided by adenosine triphosphate molecules (Dancis and Schneider, 1975).
The exact mechanisms of active transport have not yet been elucidated, but they would involve a step in which a membrane carrier, most probably a carrier-substrate complex, undergoes endogenous chemical changes linked to the availability of energy provided by ATP.

1.2.7d Endocytosis:

In this type of transport, the placenta incorporates large-molecular weight substances that cannot pass through the cell membrane pores. The substances to be transferred are taken up within invaginations of the plasma membrane, which subsequently becomes detached to form a microvesicle that is transported across the cytoplasm of the syncytium and released into the other side of the membrane.

Endocytosis involves several types of processes (Dancis and Schneider, 1975). One is phagocytosis, which for practical purposes can be defined as the incorporation of particles. The intake of extracellular fluid and large molecules is called macropinocytosis if the vesicles formed can be seen by light microscopy or micropinocytosis when the vesicles are visible only by electron microscopy.

Little is known about endocytosis in the human placenta, although it is generally believed that the most commonly used mechanism is micropinocytosis. Present knowledge on micropinocytosis derives largely from studies in endothelial cells. In this system the formation of microvesicles does not require energy, in contrast to the formation of phagocytic or macropinocytic processes (Dancis and Schneider, 1975). Immunoglobulins and other large proteins are transferred across the placenta by this process of endocytosis.
1.2.7d Bulk Flow of Ultrafiltration:

Water movement between mother and fetus is very rapid. The mechanism responsible for this rapid turnover is unknown, but it is generally attributed to hydrostatic or osmotic pressure differences. This will cause transfer of water molecules, a process referred to as bulk flow. Water movements will carry dissolved particles (solvent drag). This results in a more rapid transfer of water and solutes than that predicted on the basis of simple diffusion (Dancis and Schneider, 1975).

1.2.7e Breaks in placental villi:

A maternal-fetal exchange of substances may also take place through abnormal "breaks" or "leaks" in the placental villi. These "breaks" allow the passage of whole cells, generally from the fetus into the mother. The significance of this phenomenon in normal circumstances is unknown, but they are important in initiating sensitization in cases of Rh system or ABO maternal-fetal incompatibility (Dancis and Schneider, 1975).
1.3 AMINO ACID TRANSFER ACROSS THE PLACENTA

1.3.1 Introduction

1.3.2 Chemistry

1.3.3 Physiology

1.3.4 General principles of amino acid transport

1.3.5 Placental amino acid transporters

1.3.6 The transporters

1.3.7 Characteristics of amino acid transport systems

1.3.8 Factors regulating amino acid transport
1.3.1 Introduction:

Amino acids are required by all living cells for the replacement of structure, synthesizing essential proteins and supplying energy for metabolism. These amino acids reach the cells by traversing the membranes which form the fix boundaries of the cells. During pregnancy, the fetus is growing very rapidly and therefore requires a constant and adequate supply of amino acids, most of which are essential in utero. These amino acids reach the fetus after traversing the placental barrier. Although the study of placental amino acid transport has been predominantly in animals, most of the transporters identified in different cells or membranes and in animal placental vesicles have been identified in human placental preparations. In this chapter, basic amino acid chemistry and physiology, general principles of amino acid transport and the various transport systems currently understood as being responsible for the transport of amino acids across the human placenta will be discussed.

1.3.2 Chemistry:

An amino acid is a molecule that possesses at least one carboxyl (-COOH) group and one amino (-NH₂) group, usually bonded to the same carbon (α- or 2-atom). The other ligands of the α-carbon are - H and a side chain (R-group) of different sizes, shape and chemical properties. Except for glycine where the R-side chain is - H, the tetrahedral array of the four different ligands about the α-carbon confers specific activity on amino acids giving rise to two stereoisomers, the L- and D-forms (one being the mirror image of the other). In animal proteins, L- isomer amino acids are the main constituents. The D- stereoisomers are usually inert.
and are rapidly converted to ketoacids (Guidotti & Gazzola, 1992). D-aspartate is, however, not converted into ketoacids (Christensen, 1993).

![Amino Acid Structure](image)

Amino acids are predominantly dipolar ions (Zwitterions) at neutral aqueous solutions. In this state, the amino group is protonated while the α-carboxyl group is dissociated.

![Dissociated Amino Acid](image)

In this form, the amino acids are lipid soluble and therefore diffuse across lipid bilayers. The amino acids (most) which lack additional ionizable groups in the side chain at physiological pH are known as neutral (zwitterionic) amino acids, while those that possess a proton-accepting group in the side chain are called cationic (basic). The group that carry one or more extra carboxyl groups are called anionic (acidic) amino acids. There are 20 different amino acids and Table 1.3.1 shows the various amino acids under different groups (based on the charge on the acids at neutral pH in aqueous solution).
**Table 1.3.1**  Essential (in bold) and Non-essential amino acids (in light)

### Neutral (Zwitterionic) Amino Acids

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear aliphatic</td>
<td>Glycine, Alanine</td>
</tr>
<tr>
<td>Branched aliphatic</td>
<td>Valine, Leucine, Isoleucine</td>
</tr>
<tr>
<td>Hydroxyl-containing aliphatic</td>
<td>Serine, Threonine</td>
</tr>
<tr>
<td>ω-amides</td>
<td>Aspargine, Glutamine</td>
</tr>
<tr>
<td>sulphur containing</td>
<td>Cysteine, methionine</td>
</tr>
<tr>
<td>Aromatic</td>
<td>phenylalanine, Tyrosine</td>
</tr>
<tr>
<td>Other heterocyclic</td>
<td>Histidine, Tryptophan</td>
</tr>
<tr>
<td>Secondary</td>
<td>Proline</td>
</tr>
</tbody>
</table>

### Anionic (acidic) Amino Acids

- Aspartic acid, Glutamic acid

### Cationic (basic) Amino Acids

- Lysine, Arginine, ornithine

Most amino acids are primary amines (-NH₃⁺) but in some, one or more of the -H of the amino group is substituted by e.g. an alkyl group (e.g. methylamines); proline derivatives are examples and they are referred to as secondary amines (-NH₂⁺). The amino group in most amino acids is on the α-carbon but in some biologically important compounds, the amino group is on the β (e.g. β-alanine) or on the γ-group (for example γ-butyric acid or GABA) carbon.

Although there are 20 different amino acids which are used to build up proteins in all species, additional amino acids are found in some specialized proteins as products of post or co-translational modifications of the original amino acid
residues (e.g. hydroxyproline and hydroxylysine in collagen, $\gamma$-carboxyglutamic acid in some clotting factors, mono- and di-iodotyrosine in thyreoglonulin, etc), and free in the cytoplasm as intermediates of relevant metabolic pathways (ornithine, citrulline, homocysteine, homoserine, sarcosine etc).

1.3.3 Physiology:

Amino acids play an important role in many biological processes. These include:

1. biosynthetic precursors of proteins,
2. substrates for fuel,
3. carriers/donors of carbon and nitrogen,
4. neurotransmitters,
5. components of buffer systems,
6. compatible osmolytes.

Generally, amino acids are nutritionally clustered into 2 groups: "essential" - those that must be supplied with the diets and "non-essential" - those that may be synthesized by the mammal. The essential amino acids are highlighted in bold in Table 1.3.1. Eagle (1955, 1959) showed that cells growing in vitro, must be supplied with the essential amino acids that they are unable to synthesize or that are synthesized in amounts insufficient to sustain normal amino acid utilization and whose composition varies for different cell types. In-vivo, the interorgan cooperativity in amino acid metabolism contributes at least in part to supply amino acids to the cells thereby ensuring a distribution of these molecules among organs and tissues (Christensen et al, 1982).
Various cellular requirements of amino acids must, therefore be met by the combination of biosynthesis of some amino acids and the transport of others into the cell. Amino acid transport across the plasma membrane is therefore an important step in amino acid metabolism and must be appropriately regulated to maintain an internal pool adequate to support the activities of the cell and to assist interorgan nutrition. Fluxes and transport through intracellular membranes are required for the function of organelles and may be additional control sites for the operation of intracellular machinery.

The fetus from conception to birth is in a state of very rapid growth and metabolism. Since it depends entirely on the mother for an adequate supply of various nutrients, all amino acids may be regarded as "essential" to the fetus. To reach the fetus, they have to be transported across the membranes of the placenta- a process known to be active and energy dependent.

1.3.4 General Principles of Placental Amino Acid Transport:

The observations of Van Slyke and Meyer (1913-1914) in dogs that the free amino acid concentration in the tissues were higher than that in the plasma; and that infusing an amino acid admixture (casein hydrolysate) into these dogs raised tissue amino acid levels far more than it did the plasma levels, generated the concept of active transport. In 1917, Arthur Morse also found that the fetal plasma was richer in amino acids than the maternal plasma. He concluded from these observations that "In the face of such higher fetal values, mere diffusion could not explain the passage of amino acids through the placenta. Though the process concerned in the exchange had not been definitely classified" Morse said
that "at the present we cover our ignorance of its real nature by giving it the general name, adsorption".

Following the work of Morse (1917), Christensen and Streicher (1948) performed in-vivo studies on Guinea pigs. They studied the effect of an artificial rise (following oral loading) in amino acid concentration in maternal blood on the feto-maternal amino acid concentration ratio for glycine. Their results suggested that there was an interaction of these amino acids with a placental accumulative transport system for glycine. It was based on this experiment that the concept of amino acid transport systems was suggested. Nine years later, Page et al., (1957) made similar observations in humans when they infused pregnant women before delivery with a mixture of D and L histidine. The fact that the L-isomer was preferentially transferred faster (equilibrium reached within minutes) than the D-isomer (equilibrium reached within 3-4 hours) provided the first evidence of stereospecificity in amino acid transport. In the discussion that followed Page and others' (1957) presentation, Hughes (1957) postulated three mechanisms for the higher transfer rate of the L-isomer of the amino acid. These were:

1. The placenta by its anatomical nature could serve as a pump or accessory heart. This was based on the fact that circumferential and radial smooth muscles had been demonstrated to lie within the placenta.

2. The uterus by contracting and relaxing throughout pregnancy could exert a positive pressure, thereby pushing fluids and other substances toward the decidual-placental junction.
3. A biochemical or biochemical enzyme pumping system based on the rise in corticosteroid levels during the third trimester of pregnancy and labour. The corticosteroid he said bind the L-isomers and carry them across the placenta but not the D-isomers.

However, these three mechanisms are flawed by the argument that if they were in fact operational, then feto-maternal ratios of all substances should vary in the same manner but there are substances with higher feto-maternal ratios and others with lower feto-maternal ratios indicating that a selective transfer mechanism must exist.

It was not until the fifties that the first explicit studies on amino acid transport across the membrane were conducted (Christensen and Riggs, 1952). A breakthrough came when Oxender and Christensen (1963) formally identified systems that mediated the transport of neutral and cationic amino acids. Various groups (Kerr and Waisman, 1966; Kerr at al., 1968; Young and McFayden, 1973; Pueschel et al., 1982 and 1983) repeated the experiments of Christensen and Streicher (1948) using various labelled amino acids and made similar observations. Numerous transport systems for different groups of amino acids have today been defined and very recently some of these transport systems have been cloned using molecular biology techniques (Guidotti and Gazzola, 1992).

1.3.5 Placental amino acid transporters:

The original suggestion that specific transport mechanisms for amino acids exist in the placenta by Christensen and Streicher (1948) has now been confirmed by many workers. Despite this, placental transport of amino acids has not been as
extensively studied (in-vitro and in-vivo) as compared to transport in other tissues (Yudilevich and Swiery, 1985).

Placenta development is rapid and is accompanied by a rapidly changing metabolic activity. Much of the mass of the placenta consists of supporting structural tissues, and the trophoblast which is metabolically active represents only 13% of the tissue (Munro, 1980). Transport of amino acids from the maternal circulation will therefore either serve the high metabolic or protein synthetic function (Villee, 1977; Battaglia and Meschia, 1978; Young et al., 1982 and Bischof, 1984) of the trophoblast or will be rapidly transferred across to the fetal side to provide for fetal metabolic requirements (Battaglia and Meschia, 1978). In the placenta, the amino acids can be utilized for protein synthesis (Battaglia and Meschia, 1978; Young et al, 1982) and for its energy requirements (Yudilevich and Swiery, 1985), however, this has been shown to represent only a small fraction as most amino acids are transported into the fetal circulation (Munro et al., 1983).

1.3.6 The Transporters:

The first transporter to be defined was that for the neutral amino acids. It was known as System A (Oxender and Christensen, 1963). Since then many other transporter systems have been identified. Table 1.3.2 below shows the transport systems that have so far been identified. At least six distinct transport systems for neutral amino acids have been demonstrated (Yudilevich and Swiery, 1985).
Table 1.3.2: Amino Acid Transport Systems

<table>
<thead>
<tr>
<th>Transport System</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>System A</strong></td>
<td>This is most reactive with short, apolar or linear side chain amino acids such as alanine and glycine, amino-isobutyric and N-methylated derivatives (methyl alanine and methyl amino-isobutyric acid).</td>
</tr>
<tr>
<td><strong>System L</strong></td>
<td>This is most reactive with large, apolar branched-chain and aromatic amino acids such as leucine, isoleucine, tyrosine tryptophan, valine, phenylalanine, methionine, glutamine and non-metabolizable analogue 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH).</td>
</tr>
<tr>
<td><strong>System ASC</strong></td>
<td>Its reactivity is restricted to alanine, serine and glycine. It is distinguished from group A by its relative pH insensitivity, a higher stereospecificity and intolerance to N-methylated substances.</td>
</tr>
<tr>
<td><strong>System Gly</strong></td>
<td>This is specific for glycine and its N-methylated derivative sarcosine and is also sodium dependent.</td>
</tr>
<tr>
<td><strong>System B</strong></td>
<td>This is specific for amino acids such as β-alanine.</td>
</tr>
<tr>
<td><strong>System N</strong></td>
<td>This is sodium dependent and is specific for the nitrogen containing side chain amino acid. It is restricted to glutamine, aspargine and histidine.</td>
</tr>
<tr>
<td><strong>System y⁺</strong></td>
<td>This is ubiquitous in location and is restricted to the cationic amino acids lysine, arginine, histidine and ornithine.</td>
</tr>
<tr>
<td><strong>System X⁻</strong></td>
<td>This system is specific for the anionic amino acids and their analogues. System X⁻ AG is restricted to aspartate and glutamate, X⁻ A for aspartate and its analogues but largely excludes glutamate and its analogues while system X⁻G is restricted to glutamate and its analogues but largely excludes aspartate and its analogues.</td>
</tr>
</tbody>
</table>
Many other amino acid transport systems have been characterized but they are found mainly in other tissues such as the liver, blastocysts and the red blood cells (Guidotti and Gazzola, 1993). It is envisaged that many more transporters will be characterised with time both from the placenta and other tissues. Figure 1.3.1 illustrates the location of various amino acid transporters on the placenta.

1.3.7 Characteristics of amino acid transport systems (transporters):

Amino acid transport systems have certain characteristics that are unique to most enzymatic processes. The transporters are proteins although the structures of most of these proteins are yet to be defined.

1.3.7a Competitive Inhibition:

The first observation on competitive inhibition was made by Christensen and Streicher (1948) when they found that artificially high maternal (guinea-pig) blood levels of proline, histidine and methionine reduced feto:maternal ratios of glycine from 5:1 to almost unity, whereas glutamate did not affect the glycine ratio (Christensen and Streicher, 1948). Sybulski and Tremlay (1967) observed that alanine and serine inhibited the accumulation of glycine but not phenylalanine, histidine, lysine and glutamate in human placental sections. Enders et al. (1976), using $^{13}$C labelled amino acids including methyl aminoisobutyric acid (MeAIB) and 2 -aminobicyclo-[2, 2, 1] - heptane - 2 - carboxylic acid (BCH), showed that transport by System A was inhibited by MeAIB but not BCH; System L (phenylalanine, valine, isoleucine) by BCH and not by MeAIB and system ASC (alanine, serine, threonine and glutamine) only partially by BCH.
and MeAIB. This experiment was repeated in 1978 by Ruzycki et al and confirmed the presence of System A in the human placenta.

1.3.7b Substrate Recognition:

Recognition of a discrete group of substrate amino acids is one of the important properties of each transport system (Christensen, 1985). This substrate recognition is based on their net charge, steric conformation of the molecule, size, structure and polarity of the side chain; on the presence of alkyl substitution in the amino group; on the position of the amino group in the carbon chain and for coupled systems, on the conformational changes of the substrate-transporter complex induced by the co-substrate (Krupka, 1990).

In mammalian cells, however, there is often incomplete recognition of substrates by the receptor site of a transporter, thereby allowing considerable latitude in the structural requirements for the acceptance of an amino acid. This results in;
1. a transport system with more than one amino acid,
2. different transport systems exhibiting some degree of substrate reactivity overlapping and therefore various amino acids may be transported by more than one transport system.

1.3.7c Kinetic characteristics:

Amino acid transport is mediated by transporters. This mediated transport can be kinetically described with the same approach developed for enzyme catalysis (Christensen, 1975). In such a system, the transmembrane flux is defined by two parameters - $V_{\text{max}}$ and $K_{\text{max}}$. $V_{\text{max}}$ is the maximal rate of transport through the
system attained at infinite substrate concentration (and is a measure of the transport system's capacity) while $K_{\text{max}}$ is the substrate concentration at which the transport velocity is half-maximum. $K_{\text{max}}$ is useful in competitive inhibition experiments because when a transport system is being engaged by a substrate or a competing analogue, the simultaneous saturation effect of the inhibitor provides the related parameter often utilized in determining the concentration of the substrate needed to produce half the maximum velocity by the transport system.

The transport of amino acids by these systems is defined by a rectangular parabola because after a certain concentration, substrate transport cannot be increased. This same principle is involved in catalytic enzymatic reactions.

1.3.7d Energy Coupling:

The transport of amino acids by the various systems is mainly an active process requiring energy for the uphill movement of substrates. This energy is stored in the form of an electrochemical potential of Na$^+$ (generated and maintained by the operation of Na$^+$/K$^+$-ATPase pump). The flux of sodium down its gradient is coupled directly with the flux of an amino acid in the operation of some systems. In the case of placental amino acid transport, the Na$^+$ gradient is from maternal plasma into the trophoblastic cytoplasm.
Figure 1.3.1
Location of possible amino acid transport systems in the human placenta (the presence of more systems than are represented in the figure is likely).
CHAPTER 2

MATERIALS AND METHODS

2.1 PRINCIPLES AND MODELS OF IN-VIVO AMINO ACID TRANSPORT STUDIES AND FETAL METABOLISM

2.2 SUBJECTS AND METHODS

2.3 UMBILICAL VEIN BLOOD FLOW MEASUREMENT
2.1  Principles and Models of in-vivo Amino Acid Transport Studies and Fetal Metabolism

2.1.1  Introduction

2.1.2  Stable Isotopes

2.1.3  Gas Chromatography Mass Spectrometry

2.1.4  Arterialized heated venous sampling

2.1.5  Model for placental transfer
2.1.1 Introduction:

There are currently no in-vivo data available on amino acid transport kinetics across the placenta in human pregnancy despite the fact that several studies have looked at amino acid concentrations in timed specimens from the human neonate and fetus (Young and Prenton, 1969; Soletz et al. 1985; Economides et al., 1989; Cetin et al., 1988, 1990), maternal plasma during pregnancy (Morse, 1917; Clemetson & Churchman, 1954; Ghadimi & Pecora, 1964; Lindblad & Baldesten, 1967; Lindblad & Zitterstrom, 1968; Velazquez et al. 1976), the veno-arterial concentration differences in the cord blood and in-vitro transfer across the perfused human placenta (Schneider et al. 1979). Dynamic studies of amino acid transport across the placenta have been conducted in the pregnant guinea pig (whose haemochorial placenta is similar to that of humans) (Sparks et al. 1980) but inferences from these studies must be made with extreme caution.

The major difficulties in conducting kinetic studies across the human placenta have been those of using safe substances and establishing acceptable methodology. Firstly, radioisotope tracers which have been used in animals cannot be safely applied to humans and secondly, the studies require in-vivo measurement of umbilical blood flow, which in the past depended almost entirely on Doppler ultrasonography. With the development of stable isotopes and techniques for measuring vascular blood flow by directly using specially designed probes, it has now become possible to perform in-vivo dynamic studies of amino acid transport across the placenta in human pregnancy. In this chapter, the methodologies describing the use of stable isotopes, umbilical blood flow measurement and analytical methods of gas chromatography mass-spectrometry will be discussed. The model of human amino acid transport on which the study is based will also be discussed.
Stable isotopes were first used almost 20 years before the in-vivo application of radioactive isotopes. Schoenheimer and Rittenberg (1935) were in fact the first people to use deuterium to study fat metabolism in mice. This was soon followed by the use of $^{15}$N-labelled glycine to demonstrate the dynamic nature of the protein pool of the body (Schoenheimer et al., 1939). However, the use of stable isotopes in metabolic research was superseded by that of radioisotopes with the advent of scintillation counters and the availability of a wide variety of radioactive tracers (Wolfe, 1992). Since the 1970s, there has been a considerable growth in the application of stable-isotope tracer techniques in metabolic human research (Rennie, 1986).

Most elements exist in at least two different non-radioactive isotopic forms. Some of these isotopes are present in large proportions while others occur naturally in very small percentages (less than 10% of the total concentration of the various elements). For example carbon (C) has three isotopes namely $^{14}$C, $^{13}$C and $^{12}$C. The most abundant of these isotopes is $^{12}$C, however, in the environment, $^{13}$C occurs in very small quantities (1.08%). Such non-radioactive isotopes existing in small quantities e.g. $^{13}$C are called stable isotopes. They behave in a manner similar to that of the more abundant isotopes. However, because the mass of the stable isotope is different from that of the most abundant one, it is possible using this property to discriminate between compounds labelled with a stable isotope and chemically identical compounds labelled with the more abundant isotope. Table 2.1 shows the percent natural abundance of some stable isotopes.
Table 2.1: Percentage natural abundance of stable isotopes of carbon, hydrogen, nitrogen and oxygen.

<table>
<thead>
<tr>
<th>Element</th>
<th>Most abundant Isotope</th>
<th>Stable isotope</th>
<th>Natural abundance (Atoms % excess)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>$^{12}\text{C}$</td>
<td>$^{13}\text{C}$</td>
<td>1.080</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>$^{1}\text{H}$</td>
<td>$^{2}\text{H}$</td>
<td>0.015</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>$^{14}\text{N}$</td>
<td>$^{15}\text{N}$</td>
<td>0.370</td>
</tr>
<tr>
<td>Oxygen</td>
<td>$^{16}\text{O}$</td>
<td>$^{18}\text{O}$</td>
<td>0.200</td>
</tr>
</tbody>
</table>

From Wolfe, 1992

The advantages of stable isotopes in human investigations include:

1. They are not radioactive and therefore can be used in kinetic studies in groups such as the young, the elderly and pregnant women. By virtue of this property, they can be given in large doses (between 2-5%) to increase the sensitivity of measurements but at the same time ensuring that the normal metabolism is not perturbed. They can be used repeatedly in the same subject.

2. Oxygen and nitrogen do not exist as radioactive isotopes hence their stable isotopes can be used to study aspects of metabolism which would not be otherwise possible.
3.  Measurements with stable isotopes are more sensitive since they measure a primary event (the arrival of the atom or a molecule of a particular mass) rather than a secondary event (for example the production of a photon following the excitation of the scintillation medium).

4. Molecules can be labelled at different positions with the same or different isotopes to study intramolecular aspects of the metabolism of specific amino acids, for example the fate of an amino acid within different pathways in a specific body tissues.

The main disadvantage is that of cost. Firstly, stable isotopes, by virtue of their rarity make it difficult for them to be extracted from the environment. Secondly measurements of these isotopes require mass spectrometers/gas chromatography mass spectrometers. These instruments are expensive and require skilled technical expertise.

2.1.2b Application of Stable Isotopes:

Stable isotopes can be used in various investigations:

1. Where the use of radioisotopes is unethical (for example in babies, pregnant women and children).

2. Where for technical reasons, the investigations cannot be carried out with other tracers for example nitrogen and oxygen.

3. For investigations in which stable isotopes allow non invasive procedures with simple rapid measurements (such as breath test; Rennie et al.,1986).

4. In the investigations of complex metabolic interchanges that are not amenable to other approaches, for example amino acid metabolism (Bier, 1982, Chien et al., 1991), breath tests (Scrimgeour et al., 1986), trace

2.1.3. **Gas Chromatography Mass Spectrometry**:

2.1.3a Introduction and general principles:

Gas-Chromatography (GC) was first introduced in 1952 (James and Martin, 1952). A major breakthrough in analytical technology did not, however, occur until GC was interfaced with Mass Spectrometry (MS) to form the GC-MS system. GC separates the various biological substances while MS analysis them by their masses. GC provides a simple, rapid and reproducible method for high-resolution separation of volatile compounds. To achieve this, the sample must first be vaporized and then swept through a column (usually by a carrier gas for example helium, hydrogen, nitrogen and methane). In the column, separation occurs in a temperature controlled oven and the effluent enters the mass spectrometer interface.

In general compounds are converted to derivatives that are thermally stable, chemically inert and volatile at temperatures below 300°C before separation by GC. Derivatization is carried out primarily to improve chromatographic behaviour or detectability (Frank et al. 1982). Most metabolic substances in the body (including amino acids) are not particularly volatile because of the strong intermolecular attractions between polar groups. In order to increase their volatility, the polar groups have to be masked by derivatization. Polar groups like the N-H and O-H groups which can undergo hydrogen bonding contribute to intermolecular attraction and thus to low volatility. To increase volatility therefore, these groups are replaced by alkylation, acylation or silylation. For
example monosaccharides have little volatility; the volatility can be increased by replacing the active hydrogen with a trimethylsilyl group. There are many derivatizing methods currently available. In choosing a derivatizing method to use for GC-MS, many factors must be taken into consideration. These include ease of preparation, stability of the derivatized sample, the separation characteristics on the GC and the resulting fragmentation pattern.

2.1.3b Principles of Operation of the GC-MS

The compound to be analysed is first derivatized to render it volatile. It is then injected into the GC which has a capillary packed column. The injection port (of the GC) is generally at about 250°C so that all the samples are vaporized. The sample is then partitioned between the inert carrier gas and the stationary phase of the column. The stationary phase is a nonvolatile liquid coated onto the sides of the capillary column. More recently, however, these stationary phases are more likely to be bonded chemically. The temperature of the column can be controlled over a range of temperatures to enable the elution of compounds of interest. The sample from the column enters the ionization chamber of the MS. In the MS, the sample is bombarded with electrons which are emitted from a heated filament and attracted towards a positively charged plate or trap. When the sample is struck by an electron, sufficient energy may be imparted to remove an electron from the sample molecule resulting in a molecular ion:

\[ M + e \rightarrow M^+ + 2e \]

The electron trap is held at a potential that is positive with respect to the filament, and it is conventional to set the electron energy at 70eV (Wolfe, 1992). The molecular ion may then be fragmented by the energy in excess of that required to ionize the sample molecule. Multiple fragmentations of a molecule may occur and the pattern of this fragmentation, is reflected by the mass spectrum which is characteristic of the particular compound. Consequently the
MS of a compound can be considered to be a "fingerprint" for the purposes of identification (Figure 2.1.1 shows a typical mass spectrum for Leucine).

The ions that result after the electron bombardment, are accelerated towards the detector. The mass entering the detector is controlled by the quadrupole mass analyzer. Because of the rapidity with which data are generated from the quadrupole mass analyzer, it is interfaced with a computer for data collection, processing, manipulation and storage if necessary. The mode of data collection for the stable isotope measurements in this research was Selected Ion (one fragment containing the labelled while the other the unlabelled moiety) Monitoring (SIM). These data are presented on a spectrum as various peaks for different masses - a particular compound being identified by its spectrum (Figure 2.1.2 shows the spectrum of various D and L-amino acids).

2.1.4. Arterialized venous sampling:

In metabolic studies a steady state of various metabolites that are being investigated is required to enable easier interpretation of the kinetics of these metabolites. Within this steady state system, it is important to measure the concentrations of various substances entering and leaving the system. In the case of the feto-placental unit, this is the uterine arteries and the uterine veins. Access to the uterine arteries is difficult and to overcome this other peripheral arteries could be used. Radial or brachial arterial blood sampling at different time points would provide the levels from which the occurrence of a steady state can be determined. Sampling from these vessels is not always successful and is associated with complications such as mechanical obstruction, thrombosis and in some cases, complete loss of a limb (Machleder et al., 1972; Campion et al., 1971). In order to overcome this, Rodie and Shepherd (1957) suggested that by increasing the ambient temperature of the hand to at least 45°C, the
concentration of various metabolites in superficial venous samples will be similar to that in arteries. The heating increases blood flow, causes vasodilatation and decreases the transit time across the tissues to one third that in non-heated tissues (Hamilton and Dow, 1963; McGuire et al., 1976). The end result is that very little exchange occurs in the tissues and therefore the arterial and venous concentrations of substances is approximately equal. Abumrad et al (1981) have in fact shown that the concentrations of amino acids, lactate, free fatty acids and glycogen from heated superficial venous samples are the same as those from the radial artery. In the current study this technique was used to obtain arterialized venous samples. The samples were collected from the dorsal vein of the hand after heating in a hot box at 55-60°C for 5-10 minutes. The hot box (Figure 2.1.3) was a modified incubator.

2.1.5 A Simple Model For Placental Transfer:

The placenta is an organ which transfers various substances between two streams (maternal and fetal placental blood flows). Figure 2.1.4 shows the relationship between the maternal, placental and fetal circulation. It is therefore considered as an exchanger (Battaglia and Meschia, 1986) and a simple model for understanding the transfer across the placenta. In this model, it is assumed that there is a membrane of uniform thickness separating two compartments. One of them represents the maternal and the other the fetal compartments. Flow into the membrane from the maternal compartment carries the various substances to the placenta for exchange while flow on the fetal side carries the substances exchanged across the membrane. If the concentration of a substance $x$ in the maternal arterial blood is $Ax$ and in the maternal vein is $Vx$ then the exchanger would have removed an amount equal to $Ax-Vx$. If this substance is non-metabolizable then it would be expected that the amount getting to the fetal compartment is equal to this difference. This difference is considered as the
placental clearance of the substance. However, the amount of the substance (x) taken up by the fetus will be equal to the difference in concentration of the substance in the vessel carrying blood to the fetus (vx) and that returning from the fetus (ax) side. This will be vx-ax. If the blood flow in the artery to the exchanger from the maternal side is known, then by applying Fick's principle, fetal uptake of the substance can be calculated from the following equation:

\[ Q = F_v \times (v_x - a_x) \]

where \( Q \) = uptake, \( F_v \) = blood flow in the vessel to the fetus and \( v_x \) and \( a_x \) are the concentrations of substance x in the vessel to and from the fetus respectively. It is important that the various measurements are made under steady-state conditions for easier interpretation of exchange kinetics (Battaglia and Meschia, 1986).
Figure 2.1.1
Mass spectrum of unlabelled Leucine (Leu) and labelled Leucine (d₃Leu)
Figure 2.1.2
Spectrum of amino acids showing glycine and D & L-Leucine
Figure 2.1.3a
Hot box (modified incubator)

Figure 2.1.3b
Patient's hand and distal forearm in the hot box with seal around hand. Temperature of the box is set at 60°C.
Figure 2.1.4
The relationship between maternal and fetal circulations
2.2 PATIENTS AND METHODS

2.2.1 Patient selection

2.2.2 Recruitment

2.2.3 Preparation of the amino acids and mannitol

2.2.4 The study design

2.2.5 Processing of blood samples

2.2.6 Laboratory preparation of amino acids and mannitol

2.2.7 Calculations

2.2.8 Statistical methods
2.2.1 Patient Selection:

These were in two groups, all undergoing caesarean section (CS). The decision to perform caesarean delivery was determined clinically. The two groups were;
1. those with appropriate for gestational age fetuses,
2. those with small for gestational age fetuses.
Examples of the indications for caesarean delivery were breech presentation, two or more previous caesarean sections, previous CS and feto-pelvic disproportion, bad obstetric history, pre-eclampsia, breech presentation and small for gestational age and suspected feto-pelvic disproportion. Small for gestational age was suspected clinically when the symphysio-fundal height was more than 3 cm less than the gestational age (by early ultrasound scan) and fetal abdominal circumference on ultrasound scan was also below the 5th centile for gestational age. The charts used for this study were those of Wilcox et al., (1993) based on the population of Nottinghamshire (an adjacent county).

The study was approved by the Leicesterhire Health Authority Local Ethics Committee.

2.2.2 Recruitment:

Information sheets containing a simple and brief description of the study were prepared (Appendix 1). All patients who were booked for elective caesarean section (CS) at the Leicester Royal Infirmary Maternity Hospital between May 1993 and April 1994 were informed of the study by the doctor or midwife booking them for CS. Those who wanted more information were then seen by the author. At that consultation (which took place in the antenatal clinic or on the ward), the study was explained to the patient and her partner in simple language and diagrams were
used to help their understanding. Any questions were answered and the patient was then given the information sheet with telephone contact numbers (Appendix 2). Those who decided to take part in the study then rang back or informed the author on the day of admission, usually a day before surgery. Some of the patients, especially those who had small for gestational age fetuses were identified from ultrasound scanning performed by the author. Those who had this diagnosis were informed of the study and told that if they were to be delivered by CS, they would be invited to take part in the study.

2.2.3 Preparation of the amino acids and mannitol:

The amino acids and labelled mannitol used in the study (supplied by Tracer Technology Inc. Ithaca, Massachusetts, USA) were labelled with either $^2$H, $^{13}$C or $^{15}$N at different positions and were mixtures of D- and L-isomers in a ratio of 50:50 except glycine which is non-isomeric. They were:

Glycine [labelled with 2-$^{15}$N] - transported by System Gly
Leucine [labelled with 2-$^2$H] - transported by System L,

The amino acids were prepared on the day before surgery for elective cases and on the day of surgery for semi-emergencies. The preparation was in the intravenous additive unit (IAU) of the pharmacy department of the hospital (by Christine Clarke and Janice Lottt). On the day of admission, the patient was weighed to the nearest 0.5 kg. The amino acids were then weighed to the nearest milligram using a scale with facilities for tarring.

The total of each amino acid required per patient was calculated based on the following formula.

Bolus dose = weight of patient (kg) X 0.8 mg
Constant infusion = weight of patient X 0.8 mg X 2 hours.

This was based on the fact that the constant infusion was to run at a rate of 0.8 mg/kg/hr until the baby was delivered.
After weighing, the amino acids were dissolved in 0.9 % sodium chloride at room temperature. The mixture was allowed to stand until dissolution was complete. It was then passed through a 0.22 μm bacteriostatic filter and then drawn up into a sterile syringe using another 0.22μm filter, under aseptic techniques. The same method of preparation was employed for mannitol. Fifteen mg of $^{13}$C labelled mannitol was dissolved in 2 ml of 0.9% sodium chloride and prepared as for the amino acids.

### 2.2.4 The Study design:

Requirements:

1. Amino acids ($^2$H Labelled D- and L- Leucine and $^{15}$N labelled Glycine)
2. 10 % unlabelled mannitol (12C) and 99 % APE labelled mannitol (13C)
3. Hot box (Figure 2.1.3a)
4. Two intravenous cannulae (size 18 French Gauge)
5. Two motorized infusion pumps
6. Transonic time probe and machine
7. OSMM$^3$ Hemoximeter

An informed signed consent was obtained from each patient (Appendix 3). The patients were fasted overnight. On the morning of surgery they were transferred to the delivery suite where the study was conducted.

The hot box was pre-heated to 55°C for 30 minutes before the study was commenced. A size 18 French gauge (FG) cannula was then inserted into a peripheral vein on the right hand/arm and a three way tap connected to it. A 500 ml bag of 10% $^{12}$C mannitol was connected to the cannula through a motorized infusion syringe pump. The amino acid mixture (contained in a 60 ml syringe) was also connected through another motorized pump to the cannula. All the taps were
closed at this stage. The patient then placed her left hand in the hot box for 10 minutes (Figure 2.1.3b) after which another size 18 FG cannula was inserted into a peripheral vein on the dorsum of the hand and 5 ml of blood collected into a lithium heparinised bottle and immediately placed on ice in a polystyrene box. A bolus dose of 10% ¹²C mannitol (1.6 ml/kg) and the amino acid mixture (0.8 mg/kg) was then given through the cannula on the right hand and the time noted. The constant infusion of mannitol and amino acids was then commenced and blood samples (5 ml) collected from the vein on the left hand at 30 minute intervals until the baby was delivered. Before each arterialized blood collection, the hand was warmed in the hot box for 10 minutes (Abumrad et al. 1981).

For those patients who had their CS under an epidural anaesthesia, this was sited before the primed constant infusion but they were only topped up for surgery 30-45 minutes before the commencement of the operation. Others had their CS either under spinal or general anaesthesia given in the theatre shortly before surgery.

The CS was performed as per routine with the patient slightly tilted to the left by a wedge placed underneath her back and to the right. On opening the abdomen and reflecting the uterovesical peritoneum, the uterine vein was identified and 5 ml of blood was collected from it using a size 25 butterfly needle. As soon as the needle was withdrawn, pressure was applied onto the puncture site with a swab on a sponge holding forceps until satisfactory haemostasis was achieved. The objective of this was to minimise haematoma formation.

The lower uterine segment was next opened transversely and the amniotic sac exposed without rupturing it whenever possible. Amniotic fluid was collected into a syringe and the membranes were then ruptured. A loop of the cord was mobilised and exteriorized without delivering the baby. The transonic time perivascular probe (size 7) was then applied to the umbilical vein and blood flow measured (for
30 - 90 seconds) until flow was stable (See section 2.3). The baby was then delivered and blood was immediately obtained from the umbilical vein and artery. Fifteen miligrams of $^{13}$C labelled mannitol dissolved in 2 millitres of normal saline was next injected into the umbilical vein very close to the fetal abdomen and the baby was then separated from the cord. All these were performed while the cord was stipp pulsating. The placenta was delivered by controlled cord traction and a segment of it was dissected free washed with cold saline and stored on ice. One hour after delivery 1-2 ml of blood was collected from a superficial vein on the dorsum of the baby's hand.

2.2.5 Processing of blood samples:

All the blood samples were obtained in heparinised tubes and stored on ice until centrifuged. Shortly after collecting the samples from the cord, they were processed through a Hemoximeter on the delivery suite theatre for haemoglobin oxygen saturation ($HbO_2$SAT), carboxyhaemoglobin ($HCO$), methaemoglobin (MetHb) concentration and oxygen content. Further samples were processed in the 1306 pH/Blood Gas Analyzer (Instrumentation Laboratory) on the Neonatal Intensive Care Unit for pH, $HCO_3$, base excess and oxygen saturation. All the maternal, fetal and neonatal whole blood samples were then centrifuged at 3000 g for 15 minutes and the plasma separated and stored in Eppendorfs at -80°C until various analyses were carried out. Figure 2.2.1 shows the study protocol.

2.2.6 Laboratory preparation of amino acids and mannitol:

2.2.6a Preparation of standard working solutions:

For all the labelled amino acids used in the study, D and L (unlabelled) isomers were weighed and dissolved in distilled deionised water to form standard working
solutions. The working solutions for mannitol were $^{12}$C mannitol and 3-O-α-methyl-D-glucose (the internal standard) - both were prepared in a similar manner as the amino acid working solutions. The working solutions prepared and their concentrations were:

a) Glycine 300μg/ml

b) D - Leucine 50 μg/ml

c) L - Leucine 200 μg/ml

The internal standards used in the study included d$_{10}$ Leucine and Methyl Lysine. The working solutions for these standards were prepared as for the D and L amino acids. They were:

a) d$_{10}$ Leucine 40 μg/ml

b) Methyl Lysine 198 μg/ml

All these working solutions were aliquoted and stored at -20°C.

2.2.6b Preparation of the internal standard Curve:

Working solutions of the various amino acids to be measured (labelled and unlabelled) and the amino acid to be used as the internal standard were thawed. At the same time a 3.5 cm Dowex (Resin) column was set up in each of 7 disposable polystyrene tubes. Two ml of distilled deionised water (ddw) was then added onto the Dowex columns. The seven tubes were labelled one to seven or standard unlabelled (SUN), standard 0 (S 0), standard 10 (S 10), standard 20 (S 20), standard
30 (S 30), standard 40 (S 40) and standard 50 (S 50). Onto SUN was added 30 μl of the unlabelled D and L amino acid and the amino acid to be used as the internal standard. Thirty microlitres of the amino acid to be used as the internal standard was added to all the tubes from S0 to S50. In addition, the unlabelled amino acids were added onto each tube in increasing volume from none into S0, 10 μl in S10, 20 μl into S20 until 50 μl onto S50. The rest of the preparation was similar to that of plasma samples (see below). Table 2.2 shows a summary of this preparation. Figure 2.2.2a and 2.2.2b show the standard curves for D and L-leucine respectively.

2.2.6c Ion-exchange purification

Dowex resin (50W X-8, H+ form, 100-200 mesh, BDH, Poole, UK) was prepared for use by washing with an excess of 1M HCl and then distilled deionised water (ddw) until the supernatant was neutral. The resin was washed once with an excess of 4M ammonium hydroxide (NH4OH) before being washed again with ddw until neutral. Neutrality was determined by litmus paper. The prepared resin was stored in ddw prior to use. This extensive prewash was necessary to remove amino acids and other contaminants, introduced during the manufacturing process.
Table 2.2 Summary of the protocol for internal standard preparation

<table>
<thead>
<tr>
<th>Standard Tube</th>
<th>Standards (unlabelled) µl</th>
<th>Internal standard µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>S10</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>S20</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>S30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>S40</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>S50</td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

2.2.7 Preparation of plasma:

Dowex columns { Dowex was pipetted into a disposable polystyrene column (6 x 1 cm, Pierce, UK) to a height of approximately 3.5 cm, then wetted with 6 ml of 1M HCl prior to sample application} were set up for each fetal, neonatal and timed maternal plasma sample. Plasma samples were thawed then centrifuged at 3000 g for 3 minutes. One hundred to three hundred microlitres of each sample was pipetted onto the column. Onto each column was added 30 microlitres of the internal standard. Passing the amino acids through the resin (Dowex) enables impurities to be removed from the plasma. This is achieved by the amino acids
sticking to the resin while the column is being washed by various chemicals or ddw.

The columns were then washed with 2-3 ml of 1M HCl, followed by 5 ml of ddw. Two ml of 2M ammonium hydroxide was then added onto the column and discarded. Four ml of 2M ammonium hydroxide was subsequently added onto each column and the eluted solution containing the amino acids was next reduced to between 1.0 and 1.5 ml under liquid nitrogen (at 10 -12 psi) in a TurboVap\textsuperscript{R} LV evaporator (Zymark, Mass. USA) at a temperature of 70\textdegree C for 20 - 30 minutes. It was very important that all water was removed prior to the next derivatization step. To achieve this, the reduced volume was then aliquoted into Eppendorfs and dried down completely in the rotary evaporator (GyroVap) overnight. The Eppendorfs containing the dried down amino acids were removed from the GyroVap and derivatized.

2.2.8 Derivatization

Three hundred microlitres of 4N HCL - propanol (made of 2ml acetyl chloride and 8 ml propanol) was added to each Eppendorf and vortexed. The tubes were then incubated at 105\textdegree C for 30 minutes. The mixture was allowed to cool down and then transferred into pyrex boiling tubes and dried down under nitrogen (20 - 30 minutes). One hundred microlitres of pentofluoropropionic anhydride (PFAA) and 200 \mu l of ethylyl acetate was next added to each boiling tube which was again vortexed and then capped tightly and incubated at 130\textdegree C for 30 minutes. The mixture was allowed to cool and again dried completely under nitrogen (20 - 30 minutes ) and 1 ml of n-Heptane was then added to each tube. The mixture was transferred into auto-sampler bottles and the sample concentrated (under nitrogen) to about 100 \mu l. This was transferred to low volume inserts and the auto-sampler vials sealed tightly, ready for GC-MS analysis.
2.2.9 Gas Chromatography - Mass Spectrometry (GC-MS)

2.2.9a GC-MS methods for determining enrichment and concentration of amino acids

The concentration and enrichment of the tracer amino acids were determined by gas chromatography mass spectrometry (GC-MS). The $^{2}$H or $^{15}$N enrichment of the labelled amino acids were determined by monitoring a specific fragment (or m/z) which contained the labelled moiety. In each case, the $^{2}$H or $^{15}$N label is revealed as 1 or more atomic mass units (amu) higher than the unlabelled compound; this permits the determination of the amount of labelled material present through changes in the (M+X)$^{+}$ to (M)$^{+}$ (where X is the number of labelled moieties) ratio i.e. $^{2}$H:$^{1}$H or $^{15}$N:$^{14}$N. The ratio of $^{2}$H (labelled) to $^{1}$H (unlabelled) or $^{15}$N (labelled) to $^{14}$N (unlabelled) is calculated from the integrated area under the peak for both masses (Mathews & Hayes, 1978). Comparison of this ratio before and during infusion of tracer allows for the determination of the enrichment of the labelled (see equation 2.1).

\[
Enrichment \ (APE) = \frac{RS - RB}{1 + (RS - RB)} \times 100 \quad \text{Equation 2.1}
\]

Where, \(APE\) = atoms percent excess
\(RS\) = isotope ratio of enriched sample, and
\(RB\) = isotope ratio of unenriched (basal) sample

The concentration was determined by the addition to plasma of the internal standard (d$_{10}$ Leucine). The ratio of the total peak area of amino acid to the internal standard i.e. (M)$^{+}$ + (M+X)$^{+}$/ (M)$^{+}$ was compared with a standard curve containing
known amounts of standard treated identically to the plasma samples. Table 2.3 shows the specific masses monitored for the determination of \([^{2}H]\) Leucine and \([^{15}N]\) Glycine enrichment and concentration in plasma in this study.

Table 2.3 Specific masses used in the determination of the concentration and enrichments of Leucine and Glycine.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Specific mass monitored</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{1})H Leucine</td>
<td>235</td>
</tr>
<tr>
<td>(^{2})H Leucine</td>
<td>236</td>
</tr>
<tr>
<td>(^{14})N Glycine</td>
<td>177</td>
</tr>
<tr>
<td>d2 (^{15})Nglycine</td>
<td>179</td>
</tr>
</tbody>
</table>

Gas Chromatography - Mass Spectrometry (GC-MS) was performed on a MD 800 Fisons (Fisons, UK) operated in the selected-ion-monitoring (SIM) mode, using electron-impact (EI) ionisation (70eV). The column used for the analysis was a 30 m long, 1.0μM thick Chirasil Val column (Alltech, UK, Serial number 2336-3). The initial column temperature was 90°C. This initial temperature was held for 1 minute before being increased to 200°C (at a rate of 4°C min\(^{-1}\)) where it was held for 3 minutes prior to cooling. The injector temperature was 250°C; injection was performed in the splitless mode and the transfer line was maintained at 280°C. The carrier gas was helium (BOC, UK) and the column head pressure was set at 5-7 kPa.
2.2.10 Calculations:

Standard curves for amino acid concentration determination were prepared as described in section 2.2.6. The peak area of each unlabelled standard (M)\(^+\) + (M+1)\(^+\) was divided by the peak area of the internal standard and this ratio was plotted against the known concentration of standard. Standards of known enrichment (APE) over the range observed in plasma were prepared by mixing 99 atoms percent \(^2\)H or \(^{15}\)N labelled amino acids with known amounts of their respective unlabelled amino acid. The theoretical enrichment was then plotted against the observed/measured enrichment and regression analysis performed using a commercially available least squares fit method (QuattroPro, Borland, UK). Each sample was derivatized twice and the coefficient of variation between these measurements was 5 - 10%.

2.2.11 Statistical Analysis:

Results are presented as mean (± SD) or means (± SEM) as indicated. Statistical comparisons were performed using the Students t-test for paired samples where appropriate. Significance was assigned at the 5% level (P < 0.05) or using 95% confidence intervals where appropriate.
Primed constant infusion of sugar and amino acids

Blood samples

a) Mother

b) Baby's cord

0  30  60  90  Delivery

Figure 2.2.1
Protocol of placental transfer study
Figure 2.2.2a
Internal standard curve for L-Leucine
(Internal standard = d10 Leucine)
Figure 2.2.2b
D-Leucine internal standard curve
(Internal standard = d10 Leucine)
Only women having a Caesarean delivery can participate in the study.

All mothers have a drip during Caesarean section. As part of this study, we would like to start your drip 1-2 hours earlier. We will add to into the drip very small amounts of one or more amino acids and mannitol (which is a type of sugar). These are harmless to you and your baby.

In your other arm, we will put a small plastic needle called a cannula. This will allow us to take 4-5 small blood samples we need without using a needle each time. The samples will be taken at 20 minute intervals, and for about 10 minutes before each sample is taken, we will gently warm your arm in a box, which we will show you. Both the warming and sampling are painless.

At Caesarean section, which will be performed under epidural, another small sample of blood will be taken from a vein that comes from your womb. Your baby will have a small sample of blood taken from the umbilical cord vessels at birth. This is a normal procedure at most Caesarean sections and is a painless way of taking blood from new born babies.

At birth, a very small amount of mannitol (the sugar which is harmless) will be given to your baby through the umbilical cord. Again this is a painless procedure. One hour after delivery, a very small sample of blood will be collected from your baby's cord. The total amount of blood that would collect from you is about 15 mls (half an ounce) and from your baby is 4 mls.

Thank you for taking part in this important study. You must remember that you are at liberty at any time to change your mind and withdraw from the study.

If you have any further questions, please do not hesitate to Ask us.

David J. Taylor
Professor of Obstetrics and Gynaecology
Leicester, LE2 7LX

Dr. Justin Konje
Research Fellow

Contact phone number: Leicester 541414 Bleep 239/Air call.
STUDY OF PLACENTAL TRANSFER OF AMINO ACIDS AT CAESAREAN SECTION, FUNDED BY ACTION RESEARCH.

Dear Mrs/Miss

About 10% of all babies do not grow properly in their mother's womb. Various reasons are responsible for this growth failure but only a few have so far been identified. This study is investigating the possible reasons why there is failure to grow properly in the womb.

Everybody needs proteins to grow. Amino acids are substances that form these proteins. In this study, we are looking at how amino acids are transferred to the baby by the placenta (the "after birth"). By understanding this process, we may be able to help babies grow properly in the future. We would therefore be very grateful if you could help us help these babies that fail to grow properly by participating in this study.

Before you leave this clinic, one of us would like to speak to you about this very important study.

Thank you

Yours sincerely

[Signatures]

Professor David Taylor
Professor of Obstetrics and Gynaecology
Leicester Royal Infirmary
Leicester LE2 7LX

Contact Phone Number: Leicester 541414 Bleep No. 5214/Air call.

Appendix 2.
CONSENT FORM

I ................................................................................................................... give my consent to participate in the "Study of placental transfer of amino acids at Caesarean section". I confirm that I have:

1. Read the Patient Information Sheet,

2. Had an opportunity to ask questions and discuss this study,

3. Received satisfactory answers to all my questions,

4. Received enough information about the study,

5. Spoken to Dr. Justin Konje and

6. Understand that I am at liberty to withdraw from the study at any time without having to give a reason for withdrawing and without affecting my medical care either now or in the future.

Patients Signature.................................................................

Date..............................................................................................

Telephone Number where patient can be contacted.

Home ..................... Work ............................................

Doctor's Signature ...............................................................

Date ..............................................................................................

Doctor's contact phone number

Home ..................... Work ............................................

Appendix 3.
2.3 UMBILICAL VEIN BLOOD FLOW MEASURED BY TRANSIT TIME FLOWMETRY

2.3.1 Introduction

2.3.2 The ultrasonic *transit-time* method of blood flow measurement

2.3.3 Principles of operation

2.3.4 Subjects and methods

2.3.5 Results

2.3.6 Discussion
2.3.1 Introduction:

A variety of different methods has been used to measure umbilical blood flow in animals (Cooper et al. 1949; Dawes and Mott, 1964; Meschia et al. 1967). These have included the use of electromagnetic flow metres in exteriorized fetuses (Assali et al. 1960; Cooper et al. 1949; Dobson et al. 1966), the application of Fick's principle to the transplacental diffusion of a test substance (Meschia 1964), the steady-state diffusion method (Crenshaw et al. 1968) and the use of microspheres (Makowski et al. 1968). These methods cannot be easily applied to human beings because of difficulties of access and some of them involve administering test substances some of which are radioactive tracers and therefore unethical. Umbilical blood flow measurements in human beings have therefore been made using either the thermodilution technique (Stembera et al. 1964, 1965 & 1968; Rudolph et al. 1971; McCallum, 1977) or Doppler ultrasonography (Gill et al. 1981; Kurjak & Rajhvajn, 1982). Doppler measurements are often unreliable in practice because of difficulties in determining the angle of insonation and the diameter of the blood vessels, while thermodilution is a cumbersome and time consuming procedure.

2.3.2 The ultrasonic transit-time method of blood flow measurement:

Franklin and colleagues (1959) employed Transit-Time rather than Doppler principles to measure blood flow. In Doppler technique, flow is calculated as the product of flow velocity determined from the frequency shift of ultrasound due to reflection of sound waves from moving elements in blood and the lumenal area of the blood vessel determined from its internal diameter whereas in the ultrasonic transit-time...
technique, blood flow is determined as the product of flow velocity and the internal diameter of the blood vessel. However, the ultrasonic transit-time technique as introduced by Franklin and colleagues (1959) technique suffered from an unstable zero-flow offset (Radar, 1976). Drost (1978) in order to overcome such problems, developed an electronic circuitry system to measure flow using the ultrasonic transit-time technique, a system which overcame the unstable zero-flow offset problem and produced results which were independent of vessel diameter.

2.3.3 Principles of Operation:

In the Transonic Time system, there is a flow probe consisting of a probe body housing two transducers and a fixed acoustic reflector (Figure 2.3.1). The transducers are arranged on one side of the vessel with the reflector located on the opposite side, midway between the two transducers. The two transducers are known as the Downstream and the Upstream transducers respectively. An electrical excitation causes the Downstream transducer (Transducer 1) to emit a plane wave of ultrasound which intersects the blood vessel, is reflected by the acoustic reflector, again transects the blood vessel and is received by the Upstream transducer (Transducer 2) (Figure 2.3.2) which converts it into electrical signals. The flow meter processor attached to the probe then utilises these electrical signals to accurately calculate the transit time taken by the ultrasound wave to travel from one transducer to another. The same transmit-receive sequence is repeated but this time from the Upstream transducer to the Downstream transducer and the transit time again accurately calculated.

The vectorial motion of blood flowing through the vessel interferes with the passage of sound. During the upstream cycle, the sound wave travels
against flow and total transit time is increased over that expected in air by a flow dependent component whereas during the downstream cycle, the sound travels with flow and the total downstream transit time is shorter by the same amount. The transonic flow metre subtracts the downstream transit time from the upstream transit time utilising wide beam ultrasonic illumination. The difference between upstream and downstream integrated transit times is a measure of volume flow (Burton and Gorewit, 1984), and is independent of the diameter of the vessel over a wide range.

Each ray of ultrasonic beam undergoes a phase shift in transit time proportional to the average velocity of blood multiplied by the path length over which this velocity is encountered. With wide-beam illumination, the receiving transducer integrates the velocities over the vessel's full width and yields volume flow: average velocity multiplied by the vessel's cross sectional area. Since sampling of the transit time is at all points across the vessel diameter, estimation of volume flow measurement is independent of flow velocity profile and the fetal heart rate. Also, since ultrasonic beam rays which cross the acoustic window without intersecting the vessel do not contribute to the volume flow integral, volume flow is therefore sensed by perivascular probes even when the vessel is smaller that the acoustic window.

The ultrasonic transit time method of blood flow measurement therefore has in contrast to Doppler flow measurements the following advantages (Burton and Gorewit, 1984);

1. It is independent of the angle of insonation,
2. It is independent of vessel size,
3. It is independent of the direction of blood flow, and
4. It is not affected by the turbulence in the blood vessel or blood viscosity.

The disadvantages, however, include cost, the necessity to expose the vessels to fit the probe and the need to repeatedly sterilise the probes.

2.3.4 Subject and Methods:

The fetuses of fifty-four women undergoing caesarean delivery and who were recruited into the stable isotope amino acid protocol were studied. The indications (which are detailed in chapters 5 and 6) for caesarean delivery included breech presentation, previous caesarean section and other pregnancy complications including intrauterine growth retardation and severe pre-eclampsia. Caesarean sections were performed through a standard lower abdominal transverse skin incision. On opening the lower uterine segment, a loop of the umbilical cord was gently exteriorized and the transonic probe size 7 (Transonic Systems Inc. Ithaca, NY, USA, No HT107-92-0110) applied round the umbilical vein using sterile Aquasonic 100 ultrasound transmission gel (Parker Laboratories, Inc. Orange, N.J. USA). The application was in such a way that there was no air between the vein and the probe wall (Figure 2.3.3).

Before the caesarean section the flow metre reading was adjusted to read zero. After application of the probe, it took between 30-90 seconds before a steady reading of blood flow was established. The steady blood flow was then recorded. Figure 2.3.4 shows an integrated recording from one of the patients. The time taken from exteriorizing the cord to completing the blood flow measurement was approximately 90 seconds. The baby was then delivered.
The fifty-four fetuses were divided into 2 groups - small for gestational age and appropriate for gestational age. The criteria used to define small for gestational age are in Section 2.2. Results are expressed as means ± standard deviation (number of subjects studied). The means of the two groups were compared using student t-test, while linear regression analyses were used to determine the effects of various variables on umbilical vein blood flow.

2.3.5 Results:

Of the 54 caesarean deliveries, 33 (61%) were babies weighing over the 10th percentile for gestational age and sex while 21 (39%) weighed below the 10th percentile (using centile charts of Wilcox et al. 1993). Amongst the latter group, 15 were below the 5th percentile for birthweight (Table 2.3.1). The mean birth weight (SD) of babies weighing above the 10th centile (3227 (471)g) was significantly higher than that in those below the 10th centile (2361 (345)g) (P < 0.05).

The mean umbilical vein blood flow measured by the ultrasonic time flowmetry method in the 54 fetuses was 86 ± 24 ml. kg⁻¹ min⁻¹. Figure 2.3.5 shows that the total umbilical vein blood flow (ml. min⁻¹) measured by the ultrasonic transit time flowmetry method has a close relationship with birth weight (r = 0.89; P < 0.001). Figure 2.3.6 shows that there was a statistically significant linear correlation between umbilical vein blood flow and birth weight (r = 0.66; P < 0.004) and between blood flow and birth weight centiles (Figure 2.3.7) (r = 0.56, p = 0.003). Using simple linear regression analysis, for each 1 kg increment in birth weight, umbilical vein blood flow increased by 19.6 ± 3.3 ml. kg⁻¹ min⁻¹ (p < 0.0001; 95%
confidence interval 13.0 to 26.2). However, on multiple linear regression analysis, including gestational age in the analysis, each increment of 1 kg in birth weight was associated with an increase in umbilical vein blood flow of $21.6 \pm 5.1$ ml. kg.$^{-1}$ min.$^{-1}$ ($p < 0.001$).

The mean blood flow in AGA group ($90 \pm 18$ ml. kg.$^{-1}$ min.$^{-1}$; $n = 33$) was significantly higher ($p < 0.04$) than that in SGA group ($66 \pm 23$ ml. kg.$^{-1}$ min.$^{-1}$; $n = 21$). Blood flow was lower still in those babies weighing below the 5th percentile. However, the umbilical vein blood flow was greater than 70 ml. kg.$^{-1}$ min.$^{-1}$ in 11 of the 21 SGA fetuses. Only 3 of the 15 fetuses weighing below the 5th centile had umbilical vein blood flow rates greater than 70 ml. kg.$^{-1}$ min.$^{-1}$. Apart from 6 fetuses (with birth weight centiles between 11 - 20) in the AGA group with umbilical vein blood flow rates of between 45 and 70 ml. kg.$^{-1}$ min.$^{-1}$, all the others had flow rates above 70 ml. kg.$^{-1}$ min.$^{-1}$. In the appropriately grown preterm fetuses, the mean blood flow was 81, 93 and 115 ml. kg.$^{-1}$ min.$^{-1}$ at 34 ($n = 2$), 36 ($n = 3$) and 37 ($n = 7$) weeks respectively. These small numbers do not allow a description of the changes in umbilical vein blood flow with gestation.

2.3.6 Discussion:

Fetal growth and development depend to a large extend on an adequate supply of blood from the uteroplacental unit. In order to understand and study in any depth human fetal metabolism, a simple, non-invasive and accurate method of blood flow measurement is necessary. Measurements in the past have employed the use of substances such as nitrous oxide (Assali et al. 1960) and microspheres (Makowski et al. 1968). In some of these studies, the measurements have been made at different times during
the antenatal period and fetal weights obtained by extrapolations from growth charts (which are recognised as being inaccurate).

The *transit-time* method used in this study is simple but does require exposure of the vessel. It has been shown to be accurate in a variety of model situations (Huntington et al. 1990) where values obtained by this method corresponded very closely with measurements obtained by intra-arterial probes. In addition, preliminary comparative studies between this method of blood flow measurement and measurements obtained by the traditional Doppler ultrasound technique performed just before cesarean section showed very close correlations only when the vessel diameter was greater than 4 mm (data not included). In the absence of a 'gold' standard method for umbilical vein blood flow measurement, blood flow measured by the *transit-time* technique were compared with those determined by the Doppler ultrasound technique (Gill et al. 1974; Kurjak et al. 1982). Because it is recognised that the latter is prone to error because of difficulties in getting accurate measurements of vessel diameter (especially where vessel diameter is less than 4 mm), the Doppler measurements made, strongly correlated with those made with ultrasonic *transit-time* (where umbilical vein diameter was greater than 4 mm). The coefficients of variation of the mean values were effectively the same suggesting that the precision of the two methods are similar.

One criticism may be that the measurements were performed in conditions of altered fetal physiology. However, Stembera et al (1968) showed that during the first 3 minutes of extra uterine life, the fetal physiology is altered very insignificantly in terms of blood flow through the umbilical vein and uterine artery. Also the blood flow values in this study are close to those from studies close to term before birth measured
by the Doppler ultrasound method (Gill et al. 1981) and also those made at
delivery by the thermodilution method (Stembera et al. 1964, 1965 & 1968).
In fact the rise in blood flow up to 37 weeks and the fall thereafter is
similar to that observed by Gill et al. (1981) using the Doppler ultrasound.
The values at term are, however, slightly lower than those reported by
Eik-Nes et al. (1980) and Kurjak and Rajhvajn (1982). Possible explanations
for these differences include the effect of change in ambient temperature
on blood flow and inaccuracies in umbilical vessel diameter
measurements (up to 10 %) and antepartum fetal weight estimation in the
other studies (up to 15 - 20 %).

These results confirm those of Kurjak and Rajhvajn (1982) that blood flow
in small for gestational age fetuses is lower than that in appropriately
grown fetuses. Since blood flow through any systemic circulation is equal
to the difference between the arterial and venous pressures divided by the
peripheral resistance (Green, 1976), the results of reduced blood flow in the
small for gestational age fetuses some of whom may be growth retarded
may therefore reflect increased peripheral resistance in the placental
vessels, reduced mean arterial pressure or venous resistance. A reduction
in blood flow would be expected to be associated with a reduction in
nutrient uptake by the fetus. Since the relationship between uterine and
umbilical blood flow is direct (Assali et al. 1960), uterine blood flow in
growth retarded pregnancies with reduced umbilical vein blood flow
would be expected to be reduced. This will result in a reduction in the
supply of various nutrients including glucose, amino acids, fatty acids and
gases. The deficiency in these essential substances could therefore lead to
growth retardation.
There is increasing evidence that blood concentrations of various substances in small for gestational age fetuses are less than those in their appropriately grown counterparts. In order to properly study the uptake and metabolism of these important nutrients, a simple, safe and effective means of recording umbilical blood flow is needed. The *transit-time* Ultrasonic Volume Flowmetry which has been used very successfully in human vascular surgery (Doi et al. 1988) provides a means of measuring umbilical vein blood flow at delivery. Such flow recordings will provide the opportunity for measurements of various substances involved in metabolic exchange between the mother and fetus and uptake quotients accurately since fetal weights needed for these studies will be determined at the same time.
Table 2.3.1

Gestational age, birth weight centiles and umbilical vein blood flow. Results are presented as means and standard deviation

<table>
<thead>
<tr>
<th>Birth weight centiles</th>
<th>Birth weight centiles</th>
<th>Birth weight centiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 5th (n = 15)</td>
<td>5th - 10th (n = 6)</td>
<td>10th - 105th (n = 33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>33.1 (3.3)</td>
<td>37.7 (1.0)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1851.2 (554.9)</td>
<td>2653.3 (209.6)</td>
</tr>
<tr>
<td>Blood flow (ml. kg⁻¹. min⁻¹)</td>
<td>60 (22)</td>
<td>78 (22)</td>
</tr>
</tbody>
</table>
Figure 2.3.1
Schematic views of the perivascular transonic ultrasonic volume flow-sensor (flow probe)
Figure 2.3.2
Positioning of the transducers and the acoustic reflector round the blood vessel.
Figure 2.3.3
An intermittent (above) and integrated (below) recording from the umbilical vein
Figure 2.3.4
Ultrasonic probe showing the application of Aquasonic gel just before blood flow measurement
Figure 2.3.5
Blood flow per baby versus birth weight

$y = 27.494 \cdot 10^{0.30669x}$  \hspace{1cm} R^2 = 0.786
Figure 2.3.6
The relationship between umbilical vein blood flow and birth weight
Figure 2.3.7
The relationship between birth weight centiles and blood flow
CHAPTER 3

MANNITOL TRANSFER ACROSS THE HUMAN PLACENTA IN-VIVO
3.1 Introduction and background to the study

3.2 Subjects and methods

3.3 Samples processing and chromatography

3.4 Calculations

3.5 Results

3.6 Discussion
3.1 Introduction and background to the study:

Mannitol (C₆H₁₄O₆) is a 6 carbon hydrophilic, uncharged inert (that is it is not metabolized in the body) molecule with a molecular weight of 182. It is therefore assumed that it does not bind to maternal or fetal blood or other tissues and so should cross the placenta by a simple process of passive diffusion. Because of this property, it has been used extensively as an extracellular marker in the determination of extracellular fluid volume (space) and glomerular filtration rate (Newman et al. 1944, Dominguez et al. 1947), to measure canalicular bile flow (Erlinger & Dhumeaux, 1974), as a probe marker for intestinal permeability and clinically as an osmotic diuretic to treat conditions such as cerebral oedema (Cobden et al. 1978).

Since mannitol is thought to cross the placenta only by passive diffusion, the transport of various compounds across the mammalian placenta has been studied in-vitro using mannitol diffusion rate as the bench mark for comparison. These studies have included the transfer of various ions such as Ca²⁺ and choline, proteins, sugars and fats (Abramovich et al. 1994). Two studies have looked at the transfer of mannitol in pregnancy in-vivo (Basso et al. 1976 & Bain et al. 1988). In the former, 9 women were studied while in the latter only 6 were studied - both at the time of caesarean section. The results from these studies showed that in late pregnancy, mannitol crosses the placenta readily and subsequently appears in the amniotic fluid (Basso et al. 1976). The clearance rates across the placenta in normal term placentae was 4 - 11 ml. min⁻¹ (Bain et al. 1988). None of the two studies, however, compared transplacental clearance of mannitol in small and appropriate for gestational age pregnancies.
Given these features of mannitol transfer across the placenta, it was therefore chosen as an extracellular marker in studies of transplacental transfer of stable isotope labelled D- and L- amino acids in normal pregnancies and those with small for gestational age fetuses. Traditionally, only L-isomers of amino acids are recognised to cross the placenta by an energy dependent, ion coupling active transport mechanism while the D-isomers are believed to cross the placenta by paracellular diffusion - effectively, using the same pathway utilized by mannitol. In order to determine the relative transfer rates of the D-amino acids across the placenta, mannitol, whose clearance rate across the placenta is known and often referred to as the "gold standard" was chosen for a reference standard. It was supposed that by calculating the clearance of mannitol using well established techniques and applying this to the clearances of D-amino acids, the passive transfer rates of these amino acids could be determined.

3.2 Subjects and Methods:

Thirty - three women undergoing caesarean delivery were studied. All were fasted overnight. On the morning of surgery, each woman was given a bolus intravenous injection of ten per cent $^{12}$C mannitol (1.6 ml. kg$^{-1}$) followed by a constant infusion of 1.0 ml. kg$^{-1}$ h$^{-1}$ for 2 hours before caesarean section. Arterialized venous blood samples were collected at 30 minute intervals and processed as described in Chapter 2.

The caesarean sections were performed under either regional or general anaesthesia. Those who had caesarean section under spinal or epidural anaesthesia were pre-loaded before caesarean section. However, the concentration of $^{12}$C mannitol in maternal plasma was not different in those who had either form of anaesthesia. At caesarean section, the baby was given
15mg of $^{13}$C labelled mannitol dissolved in 2 ml of normal saline and blood collected from a superficial hand vein one hour after delivery. Attempts were made to collect urine samples from the babies during the first one hour of life but most of them (28 out of 33) did not produce any urine and those that did, produced very small volumes (less than 15 mls). It was therefore assumed that the mannitol eliminated from the neonatal body by the kidneys during the first one hour of life was small and negligible.

Small for gestational age was defined as birth weight less than the 10th centile for gestational age and gender while appropriate for gestational age was defined as birth weight above the 10th centile for gestational age and sex. The centile charts of Wilcox et al. (1993) were used.

3.3 Sample Processing and Chromatography:

Plasma from the samples was thawed and centrifuged at 3000 g for 3 minutes. Ten microlitres was pipetted into an eppendorf and 50μl of ethanol added onto it to precipitate plasma proteins. The internal standard (30μl of O-α-methylglucose) was then added. The tube was vortexed and kept on ice at 4°C for 15 minutes. The centrifuged supernatant was then dried down overnight in a rotary evaporator. One hundred microlitres of a mixture of pyridine:TMCS:BIS (volume ratio 2:1:1) was added to the dried down pellet and vortexed. The mixture was then heated at 60°C for 20 minutes. Concentration and enrichment of $^{12}$C and $^{13}$C mannitol was determined by gas chromatography - mass spectrometry (GC-MS). The $^{13}$C enrichment of mannitol was determined by monitoring specific fragments (or m/z), containing the labelled moiety, in the mass spectrum of mannitol. In each case, $^{13}$C label was revealed as 1 atomic unit (amu) higher than the unlabelled mannitol; this distinction permitted the determination of the
amount of labelled material present through the change in the (M+1)⁺ to (M)⁺ ratio i.e. ¹³C to ¹²C. The ratio of ¹³C :to ¹²C was calculated from the integrated area under the peak for both masses. Comparison of this ratio in the umbilical blood before administration of ¹³C mannitol to that in the neonatal blood allowed for the determination of enrichment of ¹³C mannitol using the following equation.

\[
\text{Enrichment (APE)} = \frac{R_S - R_B}{1 + (R_S - R_B)} \times 100
\]

Where,
\[
APE = \text{atom percent excess}
\]
\[
R_S = \text{isotope ratio of neonatal blood (enriched) sample}
\]
\[
R_B = \text{isotope ratio of umbilical vein (unenriched sample)}.
\]

The concentration was determined by the addition to the plasma of O-α-methylglycose as the internal standard.

GC-MS was performed using a MD 800 Fisons (Fisons, UK) operated in the selected-ion-monitoring (SIM) mode, using electron impact (EI) ionisation (70eV). The column used for the analysis was a SE-54 capillary column (Alltech, UK). The initial column temperature (140°C) was held for 1 minute before being increased to 280°C (at a rate of 20°C min⁻¹) where it was held for 3 minutes prior to cooling. The injector temperature was 250°C; injection was performed in the splitless mode and transfer line was maintained at 280°C. The carrier gas was helium (BOC, UK) and the column head pressure was set at 5-7kPa.
3.4 Calculations:

The clearance of mannitol was calculated from dilution of $^{13}\text{C}$ mannitol in the neonatal blood as follows:

1. Mannitol transferred to baby = $(100 \times 15) \text{ mg}$

   $\text{APE} = \text{atom percent excess (}$^{13}\text{C mannitol labelling in neonatal blood)}$

   $15\text{mg} = ^{13}\text{C mannitol given to the baby at the time of delivery}$

2. Rate of Mannitol transfer = Mannitol transferred (mg) / duration of mannitol infusion = mannitol (mg/min)

3. Mean concentration gradient of mannitol = $[\text{mean maternal conc.} - (\text{mean umbilical vessel mannitol conc.})/2] \text{ mmol/L}$ (assuming transfer was linear)

4. Conversion of mannitol conc. gradient in mmol/L to mg/ml. =  

   $\text{concentration (mmol/L)} \times 182 \times \text{MW of mannitol} / 1000$

5. Clearance (ml/min) = Total mannitol transferred (mg/min)/ mean conc. gradient (mg/ml)

Results are presented as means ± standard deviation (SD). Statistically significant differences between means were determined using paired t-test and linear relations examined by Pearson's correlation coefficient. Significance was assigned at the 5 % level ($P < 0.05$).

3.5 Results:

Of the 33 women, 20 were delivered of appropriate for gestational age babies at a mean gestational age of $38.3 \pm 1.7$ weeks while 13 had small for gestational age babies at a mean gestational age of $33.5 \pm 2.1$ weeks. The mean birth weight was $3260 \pm 447 \text{ g}$ in the AGA group and $2200 \pm 577 \text{ g}$ in the SGA group. Figure 3.1 shows a typical internal standard curve for mannitol.
assay while Table 3.1 shows the mannitol concentrations in maternal plasma at various time points during the 120 minutes of constant infusion. Figure 3.2 shows that a steady state was achieved during infusion of $^{12}$C mannitol. The average maternal mannitol concentration in the two groups is shown in Table 3.2. The concentration of mannitol in the umbilical vein and in the neonates at one hour of life are shown in Table 3.3. The labelling (APE) of the neonatal plasma with $^{13}$C mannitol was 2.8 in the AGA group and 3.2 in the SGA group (Figure 3.3). The gradient of $^{12}$C mannitol from the mother to the baby (the difference between the average maternal arterialized mannitol concentration and the umbilical vein mannitol concentration) is shown in Table 3.4.

The mean mannitol clearance across the placenta in the 33 women was $9.2 \pm 3.4$ ml. min$^{-1}$. The mean mannitol clearance of $12.8 \pm 1.6$ ml. min$^{-1}$ in the AGA group ($n = 20$) was significantly higher than that of $5.6 \pm 2.1$ ml. min$^{-1}$ in the SGA group ($n = 13$) ($P < 0.05$). Figure 3.4 shows the clearance of mannitol against birth weight. The bigger the babies, the greater the clearance of mannitol across the placenta. The relationship between mannitol clearance and maternal mannitol concentration is shown in Figure 3.5. The clearance of mannitol decreased as the maternal mannitol concentration rose. Figure 3.6 shows that there was an almost linear relationship between mannitol clearance and placental weight ($r = 0.7$, $P < 0.05$). The relationship between mannitol clearance and umbilical vein blood flow is shown in Figure 3.7. The correlation coefficient for this relationship was 0.42 ($p < 0.05$). Figure 3.8 shows that there was an inverse significant relation ($r = -0.35$, $p < 0.05$) between neonatal ($n = 27$) mannitol concentration and birthweight. There was no statistically significant correlation between mannitol clearance and gestational age ($r = 0.13$, $P > 0.05$).
3.6 Discussion:

The mean transplacental clearance of mannitol of 9.2 ml. min\(^{-1}\) in this study is similar to that of 7.3 ml. min\(^{-1}\) reported by Bain et al. 1988, however, the scatter is much wider. In the 6 cases studied by Bain et al. (1988) where the deliveries were at term the clearance rates for mannitol varied from 4.73 to 10.37 ml. min\(^{-1}\) for a birth weight variation of 2900g to 4400g. In the present study, the gestational ages at delivery varied from 32 to 39 weeks while the birth weights varied from 810 to 4700g. The variation in mannitol clearance in the present study, however, varied from 1.5 to 45 ml. min\(^{-1}\). These variations are indeed very wide. In interpreting these results and comparing them with those of Bain et al. (1988), several possible sources of error must be considered.

Methodologically, stable isotope labelling was used in this study, while Bain et al. (1988) depended on fetal mannitol excretion. The GC-MS method of mannitol assays used in the study is recognised to more accurate than the gel filtration chromatography (Laker & Mount, 1980) used by Bain et al. (1988). The assumption made in this thesis was that neonatal urine production was negligible within the first hour of life. This assumption may be incorrect as it is possible that some babies produce large amounts of urine but do not void to enable collection. If that is the case, then it is conceivable that the plasma concentration of mannitol measured at the age of one hour might have been inaccurate in some of the neonates.

A second potential source of error was the assumption that all the labelled mannitol injected into the cord at the time of delivery reached the baby. It is possible that some of it might have remained within the cord and therefore the estimation of total mannitol transferred would have been inaccurate.
Since clearance depends on the volume of plasma cleared and the concentration of the substance to be cleared in the plasma, the variation in the clearance rates of mannitol could have been influenced by these factors. In women delivering severely small for gestational age fetuses or those with pre-eclampsia, maternal plasma volume is recognised to be reduced and so too is blood flow (secondary to increased plasma viscosity and vasoconstriction). The consequences of these factors could be reduced delivery of the mannitol to the placenta and therefore a lower clearance. The studies of Bain et al. (1988) were in a homogenous population of 6 women at term and therefore these factors were unlikely to influence their results. This may be one of the reasons why their variation was not as wide.

An inverse relationship between maternal mannitol concentration and clearance was a surprised finding. Although factors such as maternal plasma volume and blood flow to the placenta may play a role in the concentration of maternal mannitol concentration these results are do not conform to the pattern expected of paracellular diffusion. The exact explanation is not immediately clear but further studies will be necessary to confirm these observations which are in fact similar to those observed by Schroder et al. (1975) for glucose transfer across the guinea pig placenta.

The significant relationship between mannitol clearance and placental weight (Figure 3.7) will suggest that mannitol transport across the placenta is also dependent to an extent on placenta size and therefore surface area. Teasdale and Jean-Jaques (1985) have shown that the diffusive area of the placenta in small for gestational age babies is reduced. This reduction is, however, not invariably associated with growth retardation. The significantly disproportionately smaller clearances of mannitol through placentas of small
for gestational age pregnancies, however, will suggest that mannitol transport across the placenta may be influenced to an extent by the morphology of the placenta.

Since mannitol clearance across the placenta was surprisingly found to be inversely related to the maternal mannitol concentration, it could not therefore be used to compare D-amino acid transfer across the placenta. The implication of these findings (if they could be replicated) is that mannitol may not be a satisfactory marker for paracellular passive diffusion. The ideal paracellular passive diffusion marker would therefore be one that is not metabolised in the body, is transported mainly by passive paracellular diffusion and is excreted freely by the kidneys without any sort of re-absorption. Antipyrine has been suggested as this ideal marker but it has been shown to be slightly metabolised in the human body (Cassady & Milstead, 1971). Inulin, another inert substance is not metabolised in the body and therefore, could be used as an ideal extracellular marker.
### Table 3.1  
Maternal arterIALIZED and umbilical mannitol concentration at different time points during infusion in 33 women.

<table>
<thead>
<tr>
<th>Time (minutes from the start of infusion)</th>
<th>Mean Mannitol concentration (mmol/L)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4.77</td>
<td>2.69</td>
</tr>
<tr>
<td>60</td>
<td>4.69</td>
<td>2.63</td>
</tr>
<tr>
<td>90</td>
<td>4.59</td>
<td>2.55</td>
</tr>
<tr>
<td>Uterine Vein</td>
<td>3.76</td>
<td>2.11</td>
</tr>
<tr>
<td>Umbilical Vein</td>
<td>3.81</td>
<td>2.04</td>
</tr>
<tr>
<td>Umbilical Artery</td>
<td>2.78</td>
<td>2.21</td>
</tr>
</tbody>
</table>
Table 3.2 Mean ± Standard deviation (SD) maternal arterIALIZED and umbilical mannitol concentration at different time points during infusion in the small (SGA) and appropriate (AGA) for gestational age groups

<table>
<thead>
<tr>
<th>Time (minutes) from the start of mannitol infusion</th>
<th>Mean (SD) mannitol concentration (mmol/L)</th>
<th>Mean (SD) mannitol concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGA (n = 13)</td>
<td>AGA (n = 20)</td>
</tr>
<tr>
<td>30</td>
<td>5.34 (3.00)</td>
<td>4.34 (2.52)</td>
</tr>
<tr>
<td>60</td>
<td>5.09 (3.03)</td>
<td>4.25 (2.37)</td>
</tr>
<tr>
<td>90</td>
<td>5.15 (2.99)</td>
<td>4.05 (2.12)</td>
</tr>
<tr>
<td>Uterine Vein</td>
<td>4.22 (2.54)</td>
<td>3.27 (1.69)</td>
</tr>
<tr>
<td>Umbilical Vein</td>
<td>3.13 (2.58)</td>
<td>2.59 (1.55)</td>
</tr>
<tr>
<td>Umbilical Artery</td>
<td>3.09 (2.78)</td>
<td>2.58 (1.71)</td>
</tr>
</tbody>
</table>
Table 3.3  Mean ± Standard Deviation (SD) mannitol concentration in the umbilical vein and neonatal blood in the small (SGA) and appropriate (AGA) for gestational age groups.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) mannitol concentration (mmol/L)</th>
<th>Mean (SD) mannitol concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SGA (n = 13)</td>
<td>AGA (n = 20)</td>
</tr>
<tr>
<td>Umbilical Vein</td>
<td>3.13 (2.58)</td>
<td>2.59 (1.55)</td>
</tr>
<tr>
<td>Neonatal Blood</td>
<td>1.77 (1.03)</td>
<td>1.50 (0.52)</td>
</tr>
</tbody>
</table>
Table 3.4  Mean ± Standard Deviation (SD) of $^{12}$C mannitol gradient across the placenta and umbilical vein $^{12}$C mannitol concentration in small (SGA) and appropriate (AGA) for gestational age groups.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) $^{12}$C mannitol concentration (mmol/L)</th>
<th>Mean (SD) $^{12}$C mannitol concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGA (n = 13)</td>
<td>Mean maternal arterialized blood sample</td>
<td>Mean maternal arterialized blood sample</td>
</tr>
<tr>
<td></td>
<td>5.20 (3.01)</td>
<td>4.21 (2.34)</td>
</tr>
<tr>
<td></td>
<td>Mannitol gradient across the placenta</td>
<td>Mannitol gradient across the placenta</td>
</tr>
<tr>
<td></td>
<td>5.21 (3.93)</td>
<td>4.37 (2.33)</td>
</tr>
</tbody>
</table>
Figure 3.1
Standard curve for mannitol using O-methylglucose
Figure 3.2
Maternal arterialized mannitol concentration during infusion.
Figure 3.3
Labelling (APE) of neonatal plasma with 13C mannitol in SGA and AGA groups
$y = -3.5477 + 6.3060 \times 10^{-3}x$  $R^2 = 0.188$

**Figure 3.4**
Birthweight versus mannitol clearance
Figure 3.5
The Relationship Between Mannitol Clearance and Maternal Mannitol Concentration.

\[ y = 34.209 - 32.469 \log(x) \quad R^2 = 0.449 \]
Figure 3.6  
Mannitol Clearance versus Placental Weight
Figure 3.7
Mannitol clearance versus umbilical vein blood flow

\[ y = -1.0935 + 0.19755x \quad R^2 = 0.177 \]
Neonatal Mannitol Concentration versus Birthweight

Figure 3.8
Neonatal Mannitol Concentration versus Birthweight

\[ y = 2.5571 - 3.2604 \times 10^{-4}x \quad R^2 = 0.126 \]
CHAPTER 4

IN-VIVO TRANSPORT OF GLYCINE BY

SYSTEM GLY

ACROSS THE HUMAN PLACENTA
4.1 Introduction

4.2 Characterisation of System Gly

4.3 Mechanism of transport by System Gly and its regulation

4.4 Background to the study

4.5 Subjects and Methods

4.6 Results

4.7 Discussion

4.8 Conclusions
4.1 Introduction:

Glycine is a non-essential, short, 2-carbon amino acid without a side chain. Its molecular weight is 75. Figure 4.1 shows the structure of glycine.

\[
\begin{array}{c}
\text{H} \\
\text{H} \\
\text{C} \\
\text{NH}_2
\end{array}
\quad
\begin{array}{c}
\text{C} \\
\text{C} \\
\text{OH}
\end{array}
\]

**Figure 4.1 Glycine**

It was the first amino acid to be isolated from proteins (hydrosylate of gelatine) by Braconnet in 1820 who called it "sucre de gelatine" because of its sweet taste. However, it was not until 1946 that its composition was determined by Horsford who then called it glycosol but the name glycine was suggested by Berzelius in 1948 (Bender, 1974).

Glycine is the only amino acid present in proteins which cannot exist in an optically active form. It is involved in a large number of important biological reactions. Glycine is an important precursor of purines and thus nucleic acids and is of importance in molecules such as adenosine triphosphate (ATP), guanidine triphosphate (GTP) and uric acid. It provides the bases ethanolamine and choline which are building blocks of phospholipids. Glycine is a direct precursor of \( \delta \)-aminolaevulinic acids and is therefore involved in the biosynthesis of haem - and therefore haemoglobin. It is also a building block for the biosynthesis of creatine,
creatinine and glutathione. In many organisms, it is used for conjugation with bile acids and foreign acids such as benzoic acids. Its metabolism is closely associated with that of serine in almost all forms of life (Neuberger, 1981), and is therefore involved in the important "one carbon" pool.

Glycine is a gluconeogenic amino acid which is also involved in a variety of synthetic reactions with protein synthesis accounting for about 50% of the metabolism of administered glycine in humans (Bender, 1974). It is a very important non-essential amino acid in protein synthesis in the body and 30% of the amino acid content of collagen is glycine (Neuberger, 1981). It therefore follows that since collagen amounts to 20-25% of the total body protein, the average glycine content of protein in the body must be fairly high. This has been estimated to be about 6% (Neuberger, 1981) which in the fetus will be almost 43% of its total protein (14% of fetal body composition is protein). Experiments conducted in rats (Neuberger, 1981) have shown that when glycine is given to pregnant rats, fetal growth is markedly improved. During the intrauterine period especially in the late second and throughout the third trimester, there is rapid fetal growth and accumulation of various nutrients. An adequate supply of these nutrients (amino acids, fatty acids and carbohydrates, gases, vitamins and minerals) is necessary for growth to occur. Glycine therefore is very important for fetal growth and development. This is because of its role in protein synthesis and as a precursor of various metabolic substances and processes. In addition, it also plays an important role in opening chloride channels in neurotransmission (Ellory et al. 1981).

Fetal requirements of glycine are in part met by maternal supply through the placenta. There is also evidence from sheep that the uteroplacental unit produces glycine from serine (Thureen et al. 1994). As a
gluconeogenic amino acid, some of it is converted to glucose which is important for brain metabolism. Excessive accumulation of glycine results in nonketotic hyperglycaemia which is a rare autosomal recessive condition while a deficiency results in disturbances in the metabolic processes (in which it takes part or forms a precursor of an intermediary substance) (Tada et al. 1992).

The concentration of glycine like that of most other amino acids is higher in the intracellular compartment than in the plasma. Since the pioneering work of van Slyke (1913-1914), it is now well established that this gradient is maintained by active transport - effected by specific transporters. In the case of glycine, this transporter is called System Gly.

4.2 Characterisation of System Gly

System Gly was first characterised in avian erythrocytes (Vidaver, 1964; Vidaver and Shepherd, 1968) but was subsequently characterised in other animal tissues such as rat hepatocytes (Kilberg and Haussinger, 1992), mouse blastocysts (van Winkle, 1992) and rat exocrine pancreatic cells. It is, however, yet to be characterised in human placental microvillous membrane vesicles (Guidotti and Gazzola, 1992).

System Gly is very limited in its substrate specificity - carrying only glycine and its N-methylated derivative sarcosine. Although Christensen (1975) suggested that System Gly may be a variant of System A, this has not been confirmed. System Gly is sodium ion (Na+) dependent and shows an absolute requirement for chloride ions (Ellory et al., 1981). The chloride dependency may also be a parallel with the role of glycine as a neurotransmitter in opening chloride channels (Ellory et al., 1981).
4.3 Mechanism of transport of System Gly and its regulation

The mechanism of transport of glycine by this system is similar to that of System A. It is energy dependent - the energy coming from the Na\(^+\) - K\(^+\) ATPase reaction. System Gly transports against concentration by coupling with Na\(^+\) transport. In this case, the intracellular Na\(^+\) concentration is lower than the extracellular concentration.

System Gly is regulated by a mechanism referred to as "adaptive regulation" or "starvation - induced transport" (Lichtenberg et al., 1983). When tissues are incubated in a glycine or sarcosine free medium, the activity of System Gly is increased (Gazzola et al., 1972). Thus, in materno - fetal transport, when fetal glycine concentration is low, placental activity of System Gly is increased. Another regulatory mechanism is that of trans - inhibition similar to that of System A. The activity of the system is inversely related to the intracellular concentration of glycine.

Although System Gly is not yet fully characterised in human placental syncytiotrophoblastic membrane vesicles, it is likely to be responsible for the transport of glycine and sarcosine across the placenta to the fetus since transport of glycine has not been shown to occur by the other well characterised placental transport systems on the placenta (Guidotti, 1993). This System Gly has similarly not been cloned, even in avian cells where it was first characterised (Guidotti, 1993).

4.4 Background to the study

The concentration of glycine in fetal plasma is greater than that in maternal plasma. The concentration of glycine in small for gestational age
fetuses especially those with hypoxia is significantly lower than that in normoxaemic small for gestational age or appropriate for gestational age fetuses (Economides et al. 1989). The feto:maternal ratio for glycine in humans varies from 1.3 to 2.5 (Linblad and Baldesten, 1967; Lindblad and Zitterstrom, 1968; Economides et al. 1989 and Cetin et al. 1990). Glycine has an important role in neurotransmission both in the central and peripheral nervous systems. The consequences of diminished levels in small for gestational age hypoxic fetuses are not well established but it is well recognised that hypoxic small for gestational age fetuses are at an increased risk of neurodevelopmental handicap (Taylor, 1989). There are two main sources of glycine to the human fetus. One is the mother, via active transport across the placenta. The other is endogenous synthesis from serine probably by the uteroplacental unit or in other fetal organs such as the liver. This has been demonstrated in ovine placentae but not yet in human placentae (Thureen et al. 1993). The diminished concentration of plasma glycine in small for gestational age fetuses could therefore be due to one of four mechanisms:

1. diminished supply from the mother to the uteroplacental unit of glycine and or serine,
2. defective glycine transport across the placenta,
3. defective production of glycine from serine, and
4. increased utilisation by the fetus for other processes (as other than protein accretion).

The studies described in this chapter were designed to test only the first two of these mechanisms.
4.5 Subjects and Methods:

Eleven women undergoing elective caesarean section were studied. The characteristics of these patients are shown in Table 4.1. The study protocol was as described in chapter 2. Each patient was given a bolus dose of 0.8 mg. kg$^{-1}$ of $^{15}$N labelled glycine intravenously followed by a constant infusion of 0.8 mg. kg$^{-1}$ h$^{-1}$ of $^{15}$N labelled glycine for 120 minutes before caesarean delivery. All the blood samples collected were processed as described in chapter 2.

The plasma samples were derivatized by using isopropanol-pentfluoropropionic acid as described in chapter 2. The internal standard used was d$_{10}$-Leucine. Gas Chromatography Mass Spectrometry was performed under the conditions detailed in Chapter 2. Figure 4.2 shows a typical standard curve for glycine while Figure 4.3 shows a gas chromatograph of labelled and unlabelled glycine.

The following equations were used to calculate the fluxes of glycine across the placenta.

\[
\text{Unidirectional flux} = \frac{\text{Enr (UmV)} - \text{Enr (UmA)}}{\text{Conc. MA} \times \text{BF}} \times \text{Enrichment (Maternal A)} \quad \text{4.1}
\]

\[
\text{Net flux} = [\text{UmV}] - [\text{UmA}] \times \text{Blood flow} \quad \text{4.2}
\]

\[
\text{Back flux} = \text{Unidirectional flux} - \text{back flux} \quad \text{4.3}
\]

The uptake of glycine by the fetus during the time of infusion was calculated using a formula based on Fick's principle.
Uptake/kg = \frac{([UmV]-[UmA]) \times \text{Blood Flow}}{\text{Birthweight (kg)} \times 1000} \quad 4.4

Fractional extraction of glycine was calculated from:

\frac{[UmV] - [UmA]}{\text{Conc.MA}} \quad 4.5

Where:

\text{Enr (UmV)} = \text{enrichment of umbilical vein by labelled glycine},
\text{Enr (UmA)} = \text{Enrichment of umbilical artery by labelled glycine}
\text{Conc. MA} = \text{Concentration of glycine in maternal arterialized vein}
\text{BF} = \text{Umbilical vein blood flow}
[UmV] = \text{Concentration of glycine in umbilical vein}
[UmA] = \text{Concentration of glycine in umbilical artery.}

4.6 Results:

Out of the 11 women, 4 were identified as carrying small for gestational age (SGA) fetuses before delivery and this diagnosis was confirmed at caesarean section while one of the seven with an antenatal classification of appropriate for gestational age (AGA) was reclassified as small for gestational age after delivery. The mean age of the women was 29.9 ± SD 6.4 years. Four of them were smokers and in none of these was a pre or post-delivery diagnosis of small for gestational age made. The indications for caesarean delivery were previous caesarean section (4), previous caesarean section and small for gestation age (3), pre-eclampsia and small for gestational age (1), pelvic kidney (1), breech presentation (1) and suspected cephalopelvic disproportion (1) (Table 4.1).

Five of the babies were below the 10th centile for gestational age and sex. The mean birth weight was 2330 ± SD 369 g and 3278 ± SD 310 g in the
small for gestational age and appropriate for gestational age groups respectively. The difference in birth weight was statistically significant ($P < 0.05$). The mean gestational ages at delivery were $34.4 \pm SD 0.5$ weeks and $38.7 \pm SD 0.8$ weeks in the small for gestational age and appropriate for gestational age groups respectively ($P > 0.05$).

The mean umbilical vein blood flow was $72.6 \pm SD 29.4$ and $84.3 \pm SD 4.1$ ml. kg.\(^{-1}\) min.\(^{-1}\) in the SGA and AGA groups respectively ($P < 0.05$).

Figure 4.4 shows the labelling [as atom percent excess (APE)] of $^{15}$N glycine in the maternal plasma during the period of infusion. Steady state labelling was achieved after priming. Because the enrichment was more than 5 APE immediately after priming, these women must have been over primed. However, this appears to have been corrected during the period of constant infusion.

The mean maternal glycine concentration in the arterialised samples in mothers carrying SGA and AGA fetuses was $245 \pm SD 84$ µmol/L and $237 \pm SD 72$ µmol/L respectively ($P > 0.05$). The mean umbilical vein and umbilical artery glycine concentrations in the AGA and SGA groups are shown in Table 4.2 and Figure 4.5. The umbilical vein concentration of glycine was similar in the two groups but the umbilical artery concentration was lower in the AGA group. Figure 4.6 shows the glycine concentration in the maternal and umbilical vein in the AGA and SGA groups. In Figure 4.7 are depicted the umbilical vein and artery glycine concentration differences. This difference was higher in the AGA group compared to the SGA group ($P < 0.05$). The umbilical vein : maternal arterialized venous glycine concentration ratio was $1.62 \pm SD 0.23$ and $1.5 \pm SD 0.18$ in the AGA and SGA groups respectively ($P > 0.05$) (Figure 4.8).
One hour after delivery, the mean neonatal plasma glycine levels was 225 ± SD 33.1 μmol/L in the AGA group and 322.4 ± SD 92.28 μmol/L in the SGA group. The levels were significantly higher in the SGA group ($P < 0.05$).

The mean uptake (Figure 4.9) by the AGA group was 2.3 ± SD 0.6 μmol. kg.$^{-1}$ min.$^{-1}$ compared to 1.2 ± SD 0.4 μmol. kg.$^{-1}$ min.$^{-1}$ ($P < 0.05$) in the SGA group. The unidirectional influx of glycine was 39 ± 14.9 μmol g.$^{-1}$ L.$^{-1}$ in the SGA group compared to 28.4 ± 10.1 μmol g.$^{-1}$ L.$^{-1}$ in the AGA group ($P < 0.05$). Table 4.3 shows that the net flux was significantly higher in the AGA group. Figure 4.10 shows the mean umbilical vein glycine concentration at birth and that in the neonatal plasma at one hour of life in AGA and SGA groups.

4.7 Discussion:

The finding of an insignificant difference in the umbilical vein glycine concentration in small and appropriate for gestational age fetuses at delivery in this study is similar to that of Cetin et al. (1988, 1990). Economides et al. (1989), however, found significantly higher glycine concentrations in the umbilical vein of appropriate for gestational age fetuses. The difference in the observations may be due to the fact that the small for gestational age fetuses in the study of Economides et al. (1989) were either at or below the third centile for gestational age while those in this study and that of Cetin et al. (1988, 1990) were at or below the tenth centile for gestational age. The maternal glycine concentration in this study was, however, higher than that in the others. This is most likely due to the fact that in this study, glycine concentration was measured in maternal arterialised venous samples which is more likely to represent
the true delivery of glycine to the feto-placental unit by the uterine artery. The concentration of amino acids in maternal venous samples which were measured in the other studies are well recognised to be lower than in the arteries.

The significantly lower umbilical veno-arterial glycine concentration difference in the SGA fetuses compared to the AGA fetuses (34.4 µmol. L\(^{-1}\) versus 87.9 µmol. L\(^{-1}\)) would indicate that the demand for glycine by the SGA fetus may not be met by uptake. Indeed the uptake of glycine in the AGA fetuses was almost doubled that in the SGA fetus (2.3 versus 1.2 µmol. kg\(^{-1}\) min\(^{-1}\)). This uptake (which better represents the amount of glycine taken up by the fetus) difference rather than the fetomaternal ratio should be used to determine differences in glycine requirements and usage between SGA and AGA fetuses. Unfortunately, uptake was not estimated in the previous studies, hence these uptake values cannot be compared.

The use of labelled glycine enabled the relative rate of transfer of glycine into the fetus to be calculated by measuring the labelling of \(^{15}\)N glycine in both maternal and fetal plasma. The enrichment of labelled glycine in the appropriate for gestational age group was 2.74 times greater than in the SGA group (P < 0.05). The net flux of glycine calculated by using \(^{15}\)N glycine was 2.5 times greater in the AGA group, however, unidirectional fluxes were higher in the SGA group. Two possible explanations can be advanced for this difference - increased transfer from the mother to the fetus (assuming that both the maternal and the fetal groups had the same enrichment at the start of infusion) or decreased turnover in the small for gestational age fetuses. The finding that the glycine concentration at one hour of life was similar to that at delivery in the small for gestational age neonates while that in AGA neonates was both lower than that at delivery
and also that in SGA neonates at the same age of life will support the latter explanation. This is consistent with the observation that gluconeogenic amino acids (such as glycine) tend to persist in the plasma of small for gestational age neonates at high levels despite hypoglycaemia (Haymond et al. 1974; Mestyan et al. 1974; Mestyan et al. 1975; Hawdon et al. 1993). Although the kinetics of transport by System Gly were not measured, the observed differences in labelling suggest that labelled glycine was transferred across the placenta more efficiently in the AGA group.

In studies performed in neonates within the first 24 hours of life Mestyan et al. (1974 & 1975) and Hawdon et al. (1992 & 1993), showed that SGA neonates have a higher concentration of gluconeogenic amino acids despite low blood glucose concentrations. In addition, infusing these neonates with gluconeogenic amino acids such as glycine and alanine has shown that while the levels in AGA babies dropped with a concomitant rise in blood glucose levels, the levels in the SGA babies remained high despite low blood glucose levels.

The alternative explanation for the persistently high plasma glycine levels in the neonates in this study one hour after birth, and persistently higher levels of glycine in SGA neonates after infusing them with gluconeogenic amino acids (Haymond et al. 1974; Hawdon et al. 1993) is that there is impaired hepatic conversion of these gluconeogenic amino acids to glucose (Mestyan et al. 1974 & 1975). However, this may not be the only explanation. It is now well recognised that an alternate metabolic pathway of glycine in birds and rats (Neuberger, 1981) is its conversion to serine. In this process, glycine undergoes direct enzymatic cleavage whereby the carbon atom of the carboxyl group is released as CO₂. Incorporation of the resultant methyl group into a second molecule of glycine forms serine.
This has, however, only been demonstrated in birds (Neuberger, 1981) or ovine fetuses (Thureen et al. 1993). Yoshida & Kikuchi (1969) showed that in a patient with hyperglycinaemia, the primary lesion was a defect of the glycine cleavage system. They proposed that the most important pathway to glycine catabolism in the human liver was direct cleavage, releasing CO$_2$ and NH$_2$. The methylene group forms methylene tetrahydrofolic acid which can react with a further molecule to form serine. The main pathway of serine metabolism in man is the cleavage by hydroxymethyltransferase to form glycine and methyl tetrahydrofolic acid. It is unlikely that there is any significant formation of serine from glycine in the human fetus. Metabolism is therefore mainly in the following pathway: Glycine $\rightarrow$ CO$_2$ + NH$_2$ + Methyl group. In addition to this, glycine takes an active part in gluconeogenesis. It enters the gluconeogenesis cycle as a source for pyruvate which is then converted to oxaloacetate (Figure 4.11). Therefore, the alternate explanation proposed here for the observed differences after birth in this study is that there could be a defect in the conversion of glycine to serine. Unfortunately, the $^{15}$N label of glycine is lost during the first step on conversion to serine and therefore it was not possible to determine the rate of glycine conversion to serine in this study. Since high levels of glycine are neurotoxic (Zitter et al. 1968) the results will suggest that supplemental feeding of SGA neonates must be balanced against the possible side effects of hyperglycinaemia. It may be speculated that the persistently high glycine (neurotoxic amino acid) levels in some SGA neonates play a role in the increased prevalence of neurodevelopmental disability in this group.
Table 4.1  Characteristics of women undergoing CS who were given 15N labelled glycine.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yrs)</th>
<th>Gest Age (wks)</th>
<th>Smoker (no/day)</th>
<th>Indication for CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>35</td>
<td>40</td>
<td>5</td>
<td>Prev. CS</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>38</td>
<td>0</td>
<td>Prev. CS</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>37</td>
<td>0</td>
<td>Pre-Ecla/SGA</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>38</td>
<td>10</td>
<td>Prev. CS</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>38</td>
<td>0</td>
<td>Prev. CS/SGA</td>
</tr>
<tr>
<td>10</td>
<td>29</td>
<td>39</td>
<td>0</td>
<td>Pelvic Kidney</td>
</tr>
<tr>
<td>11</td>
<td>33</td>
<td>37</td>
<td>0</td>
<td>Prev. CS/SGA</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>38</td>
<td>0</td>
<td>Suspected CPD</td>
</tr>
<tr>
<td>13</td>
<td>37</td>
<td>37</td>
<td>0</td>
<td>Prev. CS/SGA</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>39</td>
<td>5-10</td>
<td>Breech</td>
</tr>
<tr>
<td>17</td>
<td>35</td>
<td>38</td>
<td>20</td>
<td>Prev. CS</td>
</tr>
</tbody>
</table>

CS = caesarean section  
SGA = small for gestational age (identified before delivery)
Table 4.2

Arterialized maternal, umbilical vein and artery Glycine concentration and fractional extraction in AGA and SGA groups. Values are means and standard deviations.

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 6)</th>
<th>SGA (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal arterialized Glycine concentration (μmol. L⁻¹)</td>
<td>237.3 ± 73.0</td>
<td>245.3 ± 84.1</td>
</tr>
<tr>
<td>Umbilical vein Glycine concentration (μmol. L⁻¹)</td>
<td>368.3 ± 132.7</td>
<td>342.5 ± 104.3</td>
</tr>
<tr>
<td>Umbilical artery Glycine concentration (μmol. L⁻¹)</td>
<td>281.4 ± 118.9</td>
<td>309.4 ± 92.1</td>
</tr>
<tr>
<td>Umbilical vein minus Umbilical artery Glycine concentration (μmol. L⁻¹)</td>
<td>87.9 ± 37.4</td>
<td>34.4 ± 20.3*</td>
</tr>
<tr>
<td>Fractional extraction of Glycine</td>
<td>0.43 ± 0.16</td>
<td>0.15 ± 0.08*</td>
</tr>
</tbody>
</table>

* p < 0.05
Table 4.3
Fetal uptake of Glycine, net, unidirectional and back fluxes of glycine across the placenta. Values are means ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 6)</th>
<th>SGA (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal uptake of Glycine</td>
<td>2.26 ± 0.6</td>
<td>1.2 ± 0.4*</td>
</tr>
<tr>
<td>(μmol.kg⁻¹.min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidirectional influx of Glycine</td>
<td>28.4 ± 10.1</td>
<td>39.0 ± 14.9*</td>
</tr>
<tr>
<td>(μmol.g⁻¹.L⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net flux of Glycine across the placenta</td>
<td>13.4 ± 6.1</td>
<td>4.9 ± 2.1*</td>
</tr>
<tr>
<td>(μmol.g⁻¹.L⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backflux of Glycine across the placenta</td>
<td>14.9 ± 5.6</td>
<td>34.2 ± 18.9*</td>
</tr>
<tr>
<td>(μmol.g⁻¹.L⁻¹)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05
Figure 4.2
Internal standard curve for Glycine using d10 Leucine as internal standard
Figure 4.3
Mass spectrograph of unlabelled (Gly) and labelled (d2Gly) glycine
Figure 4.4
Labelling (APE) of maternal blood by $^{15}$N Gycine during infusion (Error bars = 1SD)
Figure 4.5
Mean umbilical vein (UmV) and artery (UmA) glycine concentrations in the AGA and SGA groups (Bars = 1SD)
Figure 4.6
Mean glycine concentration in the maternal arterialized blood, umbilical vein and artery vessels in the 11 patients (Bars = 1SD)
Figure 4.7
Umbilical vein - Umbilical artery (A-V)
Glycine concentration difference (Bars = 1SD)
Figure 4.8
Feto-Maternal Glycine concentration Ratio (Error bars = 1SD)
Figure 4.9
Uptake of Glycine in the AGA and SGA fetuses (Error bars = 1SD)
Figure 4.10
The umbilical vein (UmV) and one hour neonatal Glycine concentration (Bars = 1SD)
Figure 4.11
The gluconeogenic pathway demonstrating involvement of glycine (Gly).
CHAPTER 5

IN-VIVO TRANSPORT OF THE NEUTRAL AMINO ACID LEUCINE ACROSS THE HUMAN PLACENTA BY SYSTEM L.
5.1 Introduction

5.2 Characterization of System L

5.3 Mechanism of transport and regulation of System L

5.4 Cloning of System L

5.5 Background for the study

5.6 Subjects and Methods

5.7 Results

5.8 Discussion
5.1 Introduction:

Early in the study of amino acid transport, Christensen and Riggs (1952) showed that competitive actions among neutral amino acids were widespread in Ehrlich cells, red blood cells and the diaphragm. Cohen and Christensen (1958) also demonstrated the inhibitory effects of a variety of neutral amino acids (phenylalanine, valine, leucine, isoleucine and methionine) on the uptake of labelled valine and phenylalanine by yeast cells. Both these observations suggested that one single system was probably transporting many neutral amino acids into various cells. Heinz and Patlak (1960), however, demonstrated inconsistencies in such a hypothesis when they studied the inhibitory actions of 18 different amino acids on the initial rate of uptake of glycine into Ehrlich cells. This was based on the dissimilar degrees of inhibition by various amino acids. In some cases, the competitive actions were too small. This dissimilarity was particularly evident in cells from intestines and rat diaphragm (Akedo and Christensen, 1962). By 1963, Oxender and Christensen had concluded from various experiments that affinities for neutral amino acids clustered into two groups indicating the presence of two distinct but overlapping mechanisms. This was based on the various patterns of competitive inhibition on the uptake of these neutral amino acids. They therefore proposed the letters A and L to describe them - System A or alanine preferring or A mediation (the system that reacts most with alanine) and System L or leucine preferring or L mediation transport (the system that reacts most with leucine and phenylalanine).

5.2 Characterization of System L

Initially, it was difficult to kinetically study these systems because of substantial substrate overlapping. To overcome this, Christensen, 1965,
Christensen et al, 1965 and 1967) designed and synthesized amino acid analogues which are not metabolized by eukaryotic cells and are restricted as much as possible to a single transport system in their entry into cells. These analogues were in chronological order methyl aminoisobutyric acid (MeAIB) used as a System A specific substrate and 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) used as a System L specific substrate. In subsequent competitive inhibition studies, the component of the uptake of an amino acid that was sensitive to inhibition by MeAIB was assigned to System A and that sensitive to inhibition by BCH was assigned to System L.

The synthesis of these artificial amino acid analogues enabled further detailed characterization of the two neutral amino acid transport systems (Shotwell et al., 1983). System L is most reactive with branched-chain and aromatic amino acids such as leucine, isoleucine, valine, phenylalanine and BCH. It is Na+ independent and in some cases, is stimulated by lowered extracellular pH. It is trans-stimulated by intracellular substrates of this system (Shotwell et al., 1983). Although Systems L and A were initially characterized in Ehrlich ascites tumour cells (Oxender and Christensen, 1963), they have subsequently been demonstrated in various tissues which include human placenta (Guidotti 1993) where they have been identified on the fetal and maternal sides of syncytiotrophoblasts (Guidotti, 1993).

5.3 Mechanism of function and regulation of System L

The placenta has been demonstrated to concentrate amino acids before transferring them to the fetus. The concentration of neutral amino acids, like that of other amino acids is higher in the fetal than maternal circulation. Transport against this concentration gradient is uphill, active and energy dependent.
5.3.1 Mechanism of transport by System L

System L is sodium ion (Na⁺) independent, does not tolerate alkyl substitution for hydrogen at the α-nitrogen and has an incomplete stereospecificity (Guidotti and Gazzola, 1993). The operation of System L is independent of an electric membrane potential, being strongly stimulated by substrates present in the trans-compartment. It has a very low apparent affinity for substrate amino acids ($K_m$ of 10μM for leucine) and is unable to generate high uphill gradients for its substrates. The proposed transport mechanism for this system is a substrate-coupled antiport (tertiary active transport), whose energization is provided by the chemical gradient of transmembrane side L-reactive substrates. The inward transport of amino acids such as L-methionine and L-cysteine (site L-reactive substrates) that are concentrated intracellularly by uphill generating agencies with overlapping reactivity toward their intracellular accumulation of amino acids trans-stimulate the activity of System L, and by exchange promotes the inward movement of other amino acid substrates of this agency.

5.3.2 Regulation of System L

The regulation of the activities of amino acid transport systems enables various cells to adapt to changes in nutrient availability. Gazzola et al. (1972) first observed that incubation of certain tissues in amino acid free medium for 3 - 5 hours led to increased neutral amino acid transport. This response has been referred to as adaptive regulation or starvation-induced transport enhancement (Lichtenberg et al. 1983). The starvation induced transport is accompanied by an increase in $V_{max}$ of uptake of amino acids by System A with no significant change in the $K_m$ of uptake (Gazzola et al., 1972; Dall' Asta
et al. 1978). Another related mechanism by which System A is regulated is trans-inhibition. This is a regulatory mechanism in which the higher the concentration of the substrated amino acid transported into the cells, the lesser the activity of the transporter system.

Unlike System A, the activity of System L is either unchanged or is only slightly decreased following complete starvation for amino acids (Heaton & Gelehrter, 1972; Guidotti et al. 1975; H Kelley & Potter, 1979). The regulation of the activity of transport by System L, however, appears to be linked to the availability of leucine for protein synthesis and conceivably the signal for this leucine dependent regulation may be the ratio of charged to uncharged tRNA\textsubscript{leu}, rather than simply the size of intracellular pool of leucine (Shotwell et al., 1983). The activity of transport of System L decreases significantly in cells depleted of amino acids and increases in those pre-loaded with L site-active amino acids (Heaton and Gelehrter, 1977).

5.4 Cloning of System L:

The first attempt to clone an amino acid transport system was made in 1988 by McCormick et al. They used immunoprecipitation and membrane protein fractions enriched for System A (assayed by reconstitution with proteoliposomes). A 120 - 130 kDA peptide thought to be a component of System A was identified in Ehrlich ascites tumour cells. Recently, McCormick and others (1991) by using radiation inactivation of System A transport activity have supported their 1988 identification and assign a r.m. of approximately 350kDA to System A transporter in Ehrlich cell plasma membrane. However, cloning of this membrane transport protein in human placenta is yet to be achieved.
Tate et al. (1992), using the *Xenopus* oocyte library for the transport of branched or ring amino acids, have obtained a 2.3 kilobase cDNA clone that encodes for a protein of 683 amino acids (with 4 putative membrane spanning domains). The expression product, however, exhibits many, but not all the properties of a classic L-system transporter and may be one of the L-type amino acid transporter with overlapping substrate specificities that are expressed in different tissues. Although no definite cDNA which encodes for a protein with characteristic expression product of *System L* has been produced, it is envisaged that this will very soon be identified (Guidotti, 1993). Presently this system has not been cloned in the human placenta.

5.5 Background to the study

Leucine is a branched chain essential amino acid with a molecular weight of 131 (Figure 5.1). It makes up 8% (Gelfand and Barrett, 1987) of the 14% total protein in the human fetus (Widdowson et al. 1979). There is evidence to show that net placental supply of leucine exceeds fetal demand (Chien et al. 1993), therefore leucine supplied to the fetus is not only utilized for protein accretion but for oxidation as metabolic fuels and starting material for other amino acids.

\[
\text{CH}_3 \\
\text{CH}_3\text{---CH---CH}_2\text{---CH---COOH} \\
\text{NH}_2
\]

*Figure 5.1 L-Leucine*
Leucine metabolism occurs predominately in muscle. It can be transaminated into α-ketoisocaproate, α- keto-β-methylvalerate and α-ketoisovalerate or into α-ketoglutarate and glutamic acid. The ketoglutarate is then further converted into alanine while the glutamic acid is either converted to glutamine after combining with an amino group or is incorporated to form pyruvate. In the muscle, therefore, leucine is utilized not only for the building up of proteins but for energy production, synthesis of alanine and pyruvate. The ketoacids formed from the branched chain amino acids can be completely degraded to carbon dioxide with the liberation of energy as ATP.

Branched chain amino acids are not concentrated by the liver because the later is relatively ineffective at oxidising these amino acids. They are therefore taken up by muscle where they are oxidised. In the human fetus, therefore, storage of these amino acids is limited. However, as an important building block for muscle protein and metabolism, adequate fetal demands must be met by maternal supply. The concentration of leucine is higher in the fetal plasma than in the maternal plasma (Ghadimi and Pecora, 1964; Young & Prenton, 1969; McIntosh et al. 1984; Cetin et al. 1988 & 1990; Economides et al. 1989; Chein et al. 1993). The feto:maternal (F:M) ratio is therefore always greater than one.

Various studies have demonstrated that the F:M concentration ratio of leucine is reduced in small for gestational age fetuses (Cetin et al. 1988, 1990; Economides et al. 1989). This reduction could be due to an increase in feto-placental consumption of leucine or a placental dysfunction associated with inadequate delivery of leucine across the placenta. The recent observations that the safety margin by which leucine delivery exceeded the requirements of the fetus for protein accretion is relatively small (Chien et al. 1993) would
suggest that a decrement in placental capacity for leucine transport would impinge upon the ability of the fetus to grow adequately. Furthermore, because leucine and other amino acids are a major source of substrate for fat synthesis and make up a much larger contribution to the supply of fuel for energy production than in postnatal states (Battaglia & Meschia, 1986), limitations of placental amino acid transfer capacity could have wide ranging consequences for fetal growth and development.

Despite the evidence that the feto-maternal concentration ratio of leucine is lower in small for gestational age fetuses than in appropriate for gestational age fetuses (Cetin et al. 1988, 1990), there are currently no studies that have examined the flux of leucine from the mother to the fetus; thus the question of whether or not the lower fetal branched chain amino acid (leucine) concentration represents increased fetal utilization or decreased transfer has not be answered. This chapter describes studies in which measurements of transplacental fluxes of leucine were made in an attempt to answer the question "is the lower feto-maternal concentration ratio for leucine in small for gestational age pregnancies due to decreased transplacental transfer?"

5.6 Subjects and Methods

Fourteen women undergoing caesarean delivery were studied. The indications for caesarean section (CS) were previous CS (7), breech and small for gestational age (3), pre-eclampsia and small for gestational age (2), pre-eclampsia and diabetes mellitus (1) and breech presentation (1). The diagnosis of small for gestational age was made antenatally in 5 cases. Table 5.1 shows the various characteristics of the 14 women.
The patients were prepared for surgery and the study as described in Chapter 2. Each was infused with $^2$H labelled leucine (Mass Trace Inc. Somerville, Massachusetts) at a dose of 0.8 mg. kg$^{-1}$ for the bolus infusion and 0.8 mg. kg$^{-1}$ h$^{-1}$ for the constant infusion. The duration of constant infusion was two hours. Blood samples were collected and processed as described in Chapter 2. The plasma samples were derivatized with pentofluoropropionic anhydride (using d$_{10}$ leucine as the internal standard) as described in Chapter 2. Figure 5.2 (a & b) show typical internal standard curves for D- and L- leucine.

Fetal uptake of leucine and various fluxes across the placenta were calculated as detailed in section 4.5 of Chapter 4.

5.7 Results

Of the five cases suspected as being small for gestational age (SGA) antenatally, 4 were confirmed as SGA at caesarean section. Two of the babies that were SGA were not identified antenatally. There were therefore two groups of patients - one with appropriate for gestational age (AGA, n = 8) and the other with small for gestational age (SGA, n = 6) babies. The mean gestational age was 37.8 ± 2.5 weeks and 36 ± 3.2 weeks ($P > 0.05$) in the AGA and SGA groups respectively. The mean ± SD birthweight in the AGA group was 3031.3 ± 398 grams compared to 2140 ± 310 in the SGA group ($P < 0.05$). The mean ± SD umbilical vein blood flow (Table 3.2) was 79 ± 7.4 ml. kg$^{-1}$ min.$^{-1}$ in the AGA group and 66.8 ± 9.3 ml. kg$^{-1}$ min.$^{-1}$ in the SGA group ($P < 0.05$). Table 5.3 shows the mean maternal weights, birthweight and placental weights in the AGA and SGA groups.

The labelling (APE) of maternal plasma with $^2$H leucine during the period of infusion is shown in Figure 5.3 and confirms that a steady state was achieved. The slight drop in the APE (atom percent excess) before CS reflects the time
during which patients were infused with fluids before spinal anaesthesia or the final epidural anaesthetic top-up for surgery.

Table 5.4 shows the concentration of L-leucine in the maternal arterIALIZED venous blood of the AGA and SGA groups. There was no statistically significant difference ($P > 0.05$) in the mean maternal L-Leucine concentration in the two groups. The fractional extraction of L-Leucine (Figure 5.4a) was significantly higher in the AGA group ($P < 0.05$). The mean umbilical vein L-Leucine concentration was slightly higher in the AGA group (Figure 5.5a) but this difference was not statistically significant ($P > 0.05$). Conversely, the mean umbilical artery L-Leucine concentration was slightly (but not significantly) lower in the AGA group ($P > 0.05$). However, the umbilical veno-arterial L-Leucine concentration difference (Figure 5.6a) in the AGA group was significantly higher ($P < 0.05$) than in the SGA group. The feto:maternal ratio of L-Leucine (Figure 5.7a) was, however, not different in the two groups. The labelling of the umbilical vein by $^2$H Leucine (Figure 5.8) was also significantly higher in the AGA group.

Table 5.5 shows that the mean D-Leucine concentration in the umbilical vein was higher than that in the arterialized maternal vein; the concentration of D-leucine in the maternal arterialized vein was lower in the AGA group compared to that in the SGA group ($P > 0.05$). The mean umbilical vein and artery D-Leucine concentration was again lower in the SGA group (Figure 5.5b). However, the veno-arterial concentration difference (Figure 5.6b) was significantly higher in the AGA group ($P < 0.05$). The feto:maternal ratio of D-Leucine was not different in both groups (Figure 5.7b).

The fractional extraction of D-leucine (Figure 5.4b) was significantly higher in the AGA group (0.45) compared to the SGA group (0.23) ($P < 0.05$). Despite
the concentration of D-leucine in the maternal plasma being a tenth of that of L-leucine, the fractional extraction of D-leucine was virtually identical (Figures 5.4a and 5.4b) in the AGA and SGA groups to that of L-leucine. D-leucine was concentrated by the placenta to the same extent as L-leucine, so that the feto-maternal concentration ratios of D-, and L-leucine were identical; nevertheless, the fractional extractions of D-leucine were twice as great in AGA fetuses than in the SGA group.

The net uptake of L-leucine (Figure 5.9a) was significantly higher in the AGA group (2.34 ± 0.5 μmol. min.-1 kg⁻¹) than that in the SGA group. Similarly, D-leucine uptake was significantly higher (P < 0.05) in the AGA group (0.8 ± 0.2 μmol. min.-1 kg⁻¹) (Figure 5.9b) than in the SGA group. The unidirectional flux of L-leucine was also significantly greater in the AGA than in the SGA fetuses (Table 5.6). This was due to the fact that whereas the net flux of L-leucine was significantly greater in the AGA group, the backflux of L-leucine was significantly greater in the SGA group. The total net uptake of D-leucine in the SGA fetuses was two thirds that in the AGA group.

Neonatal D-Leucine concentration at one hour of life was determined in only 6 of the 14 babies.

5.8 Discussion

The results failed to demonstrate any significant difference in the feto-maternal (F:M) concentration ratio of both D- and L-leucine. In fact the F:M ratios were lower than those reported in previous studies (Cetin et al. 1988, 1990). The possible explanations for the differences in the F:M ratios could firstly be the that numbers in this study are small and secondly that leucine concentrations were measured in maternal arterialized venous samples.
Amino acid concentration in these samples reflect levels in the uterine artery and it is well recognised that the concentrations of these amino acids in arteries are higher than in veins. Since previous studies measured maternal venous samples, the lower values obtained from them will result in a higher F:M ratio.

The observed diminished net leucine uptake in small for gestational age fetuses (0.8 vs 2.34 µmol. min.\(^{-1}\) kg.\(^{-1}\)) suggests that the narrow safety margin identified by Chien et al. (1993) is altered in small for gestational age pregnancies. The result is an imbalance which effectively results in a diminished supply to the fetus of leucine necessary for protein accretion, building of muscle bulk and inadequate fat synthesis. This is probably one of the mechanisms contributing to poor intrauterine growth. In the AGA fetuses, the net uptake values for L-leucine were similar to those reported by Chien et al. (1993). These uptake values rather than plasma concentrations more appropriately reflect differences in leucine transfer and metabolism in AGA and SGA fetuses. In addition, the marked differences in the fractional extraction of L-leucine in both groups suggest differences in metabolic rates. The high back flux of leucine in the SGA group would be consistent with diminished utilization in this group of fetuses.

Since leucine is transported by System L across the placenta, could the diminished uptake be related to transport to the fetus? In this study, the function of System L was not directly studied, however, the physiological function of this system was indirectly assessed using plasma labelling with \(^2\)H leucine. The lower \(^2\)H leucine labelling (APE) in the umbilical vein of small for gestational age fetuses suggests a diminished rate of transfer. The exact mechanism by which this alteration in placental transport occurs is yet to be elucidated. Such a transport defect will explain the observation of

The exact mechanism by which amino acid transporters are produced and regulated is increasingly being unravelled by new molecular biology techniques (Guidotti, 1993). Defective transport of leucine across the placenta could occur at different levels. Firstly, it could be genetic, that is the genes for System L may be abnormal. If this is the case, then there may be a subset of System L which is structurally different from normal System L. Secondly, the trophoblastic cells of the placenta which produce System L may be damaged by various insults such as hypoxia with a resultant diminution in the production of System L. In order to understand and determine the level at which these defects may occur, the amino acid sequence of System L must first be determined. Following this, quantitative and qualitative studies should be conducted. Since there is now considerable progresss in cloning the various amino acid transporter, such studies should soon be possible.

The finding of a maternal arterIALIZED venous D leucine concentration consistently lower than the umbilical vein value was a surprised finding. This suggests that contrary to previous evidence (Page et al. 1957), D-amino acid transport is active and not passive. Indeed the results showing that the fractional extraction of both D- and L-leucine were similar despite the concentration of D-leucine being one tenth that of L-leucine suggest that the same transporter could be responsible for the transport of D- and L- isomers of leucine. In fact, the fractional extraction of D- and L-leucine in SGA pregnancies was always half that in the AGA group. The similarity of this relative proportionality seems to suggest the same transporter whose activity is reduced in SGA pregnancies. It is speculated that System B°+ which is
sodium dependent and involved in concentrative transport of L-leucine (Bertran et al. 1992; Mackenzie et al. 1994) may not be stereospecific as it may also transport D-leucine. The transporter's activity appears to be diminished in SGA pregnancies.

D-amino acids have been assumed to cross the human placenta passively. This was first suggested by Page et al. (1957) after infusing pregnant women with D- and L- histidine. Recently, however, Christensen (1990) has questioned the rational for this observation with particular reference to D-Leucine. Guidotti & Gazzola (1992) similarly stated that the stereospecificity ascribed to the amino acid transport systems may not be true for System L - the leucine transporter. The fact that maternal arterialized blood D-leucine concentration in this study was always lower than that in the umbilical vein suggests an active transport mechanism. Since this study did not use BCH (the paradigm substance for System L) it can only be speculated that based on the comments mentioned above, this system may in fact transport both D- and L-isomers of leucine. On the other hand, if the transporters are stereospecific then a separate transporter may exist for the D-amino acid. There is as yet no evidence to suggest either mechanism of transport for the D-isomer of leucine.

D-amino acids occur in very small and insignificant proportions in various foods (Christensen 1993). Their exact role in amino acid metabolism is unclear, however, there are specific enzymes in the body which convert these amino acids into ketoacids. These enzymes are referred to as the D-amino oxidases (Nagata et al. 1989). The higher concentration of D-leucine in the plasma of AGA fetuses both at delivery and at one hour of life also indicate that the function of the D-amino acid transporter, if it is indeed different from that of the L-isomer is also altered in SGA pregnancies. Similarly, a reduced
uptake of this isomer in SGA fetuses and yet a similar concentration in the neonatal plasma at one hour of life in the AGA and SGA groups may indicate defective conversion by D-amino oxidases in the SGA neonates. However, because D-leucine was determined in only six neonates, this can only be speculative.

In conclusion, these findings suggest that the previously reported lower concentration of leucine in the plasma of SGA fetuses may be accountable for by reduced uptake and diminished transporter activity and a high backflux into the maternal circulation.
Table 5.1
Indications for Caesarean delivery in 14 women infused with $1^{2}H$ labelled Leucine.

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Indication for CS</th>
<th>Gestation</th>
<th>Cigarettes/day</th>
<th>BWT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 previous CS</td>
<td>40</td>
<td>5</td>
<td>3330</td>
</tr>
<tr>
<td>2</td>
<td>previous CS</td>
<td>40</td>
<td>5</td>
<td>3040</td>
</tr>
<tr>
<td>3</td>
<td>2 previous CS</td>
<td>38</td>
<td>0</td>
<td>3230</td>
</tr>
<tr>
<td>4</td>
<td>PET/SGA</td>
<td>37</td>
<td>0</td>
<td>1890</td>
</tr>
<tr>
<td>5</td>
<td>2 previous CS</td>
<td>38</td>
<td>10</td>
<td>2780</td>
</tr>
<tr>
<td>6</td>
<td>3 previous CS</td>
<td>38</td>
<td>15</td>
<td>3400</td>
</tr>
<tr>
<td>7</td>
<td>3 previous CS</td>
<td>38</td>
<td>25-10</td>
<td>2770</td>
</tr>
<tr>
<td>14</td>
<td>Breech</td>
<td>41</td>
<td>5-10</td>
<td>3150</td>
</tr>
<tr>
<td>40</td>
<td>Breech/SGA</td>
<td>36</td>
<td>0</td>
<td>1910</td>
</tr>
<tr>
<td>41</td>
<td>2 previous CS</td>
<td>38</td>
<td>10-15</td>
<td>3020</td>
</tr>
<tr>
<td>42</td>
<td>Breech/SGA</td>
<td>38</td>
<td>5-20</td>
<td>2960</td>
</tr>
<tr>
<td>43</td>
<td>PET/SGA</td>
<td>30</td>
<td>0</td>
<td>0840</td>
</tr>
<tr>
<td>44</td>
<td>Breech/SGA</td>
<td>37</td>
<td>20</td>
<td>2650</td>
</tr>
<tr>
<td>45</td>
<td>Diabetes/PET</td>
<td>34</td>
<td>0</td>
<td>2120</td>
</tr>
</tbody>
</table>

CS = caesarean section; SGA = Small for gestational age; PET = pre-eclampsia
Table 5.2
Umbilical vein blood flow in AGA and SGA groups. Values are means and standard deviations (SD)

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 8)</th>
<th>SGA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total umbilical vein blood flow (ml. baby⁻¹ min⁻¹)</td>
<td>245.1 (45)</td>
<td>161.3 (43.1)*</td>
</tr>
<tr>
<td>Umbilical vein blood flow per kg birth weight (ml. kg⁻¹ min⁻¹)</td>
<td>79.0 (7.4)</td>
<td>66.8 (9.3)</td>
</tr>
<tr>
<td>Umbilical vein blood flow per kg of placenta (ml. kg⁻¹ min⁻¹)</td>
<td>448.2 (29)</td>
<td>190.8 (114)**</td>
</tr>
</tbody>
</table>

* p < 0.05  
** p < 0.005
Table 5.3
Maternal weight (kg), birth weight (g) and placental weight (g) in AGA and SGA groups. Values are means and standard deviations (SD).

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 8)</th>
<th>SGA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal weight</td>
<td>74.4 (6.5)</td>
<td>69.3 (9.8)</td>
</tr>
<tr>
<td>Birth weight</td>
<td>3031 (398)</td>
<td>2140 (310)*</td>
</tr>
<tr>
<td>Placental weight</td>
<td>547.9 (27.2)</td>
<td>378.3 (179)**</td>
</tr>
</tbody>
</table>

*p < 0.05  
**p < 0.001
Table 5.4
Concentration and labelling with deuterium L-Leucine in maternal and umbilical vessels and fractional extraction of L-Leucine in AGA and SGA groups. Values are means and standard deviation (SD).

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 8)</th>
<th>SGA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal arterialized L-Leucine concentration (µmol. L⁻¹)</td>
<td>86.8 (22.4)</td>
<td>91.0 (14.0)</td>
</tr>
<tr>
<td>Umbilical vein L-Leucine concentration (µmol. L⁻¹)</td>
<td>121.1 (21.8)</td>
<td>116.0 (25.0)</td>
</tr>
<tr>
<td>Umbilical artery L-Leucine concentration (µmol. L⁻¹)</td>
<td>91.8 (21.5)</td>
<td>103.0 (26.0)</td>
</tr>
<tr>
<td>Umbilical vein minus umbilical artery L-Leucine concentration (µmol. L⁻¹)</td>
<td>35.4 (17.3)</td>
<td>14.7 (6.8)*</td>
</tr>
<tr>
<td>[²H]-Leucine labelling I arterialized maternal blood (APE)</td>
<td>4.1 (1.0)</td>
<td>3.4 (1.2)</td>
</tr>
<tr>
<td>[²H]-Leucine labelling of umbilical vein (APE)</td>
<td>1.8 (1.1)</td>
<td>1.3 (0.5)</td>
</tr>
<tr>
<td>Fractional extraction of L-Leucine</td>
<td>0.34 (0.18)</td>
<td>0.15 (0.09)*</td>
</tr>
</tbody>
</table>

* p < 0.05
Table 5.5
Arterialized maternal, umbilical vein and artery D-Leucine concentration and fractional extraction in AGA and SGA groups. Values are means ± standard deviation (SD)

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 8)</th>
<th>SGA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal arterialized D-Leucine concentration (μmol. L⁻¹)</td>
<td>8.7 (2.6)</td>
<td>11.3 (0.98)</td>
</tr>
<tr>
<td>Umbilical vein D-Leucine concentration (μmol. L⁻¹)</td>
<td>14.1 (4.8)</td>
<td>16.4 (3.7)</td>
</tr>
<tr>
<td>Umbilical artery D-Leucine concentration (μmol. L⁻¹)</td>
<td>10.4 (14.5)</td>
<td>14.0 (2.7)</td>
</tr>
<tr>
<td>Umbilical vein minus umbilical artery D-leucine concentration (μmol. L⁻¹)</td>
<td>4.3 (31.3)</td>
<td>3.4 (0.6)</td>
</tr>
<tr>
<td>Fractional extraction of D-Leucine</td>
<td>0.45 (0.22)</td>
<td>0.23 (0.12)*</td>
</tr>
</tbody>
</table>

* p < 0.05
Table 5.6
Fetal uptake of D- and L-Leucine and net and unidirectional fluxes of L-Leucine in AGA and SGA groups. Values are means ± standard deviation (SD)

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 8)</th>
<th>SGA (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total net fetal uptake of L-Leucine (µmol. kg⁻¹ min⁻¹)</td>
<td>2.34 (0.5)</td>
<td>0.8 (0.2)*</td>
</tr>
<tr>
<td>Total fetal uptake of D-Leucine (µmol. kg⁻¹ min⁻¹)</td>
<td>0.30 (0.1)</td>
<td>0.21 (0.1)</td>
</tr>
<tr>
<td>Unidirectional flux of L-Leucine across the placenta (µmol. g⁻¹ L⁻¹)</td>
<td>0.8 (0.2)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>Unidirectional influx of L-Leucine across the placenta (µmol. kg⁻¹ L⁻¹)</td>
<td>4.31 (1.1)</td>
<td>2.5 (0.7)</td>
</tr>
</tbody>
</table>
Figure 5.2a
Internal standard curve for L-Leucine
(internal standard = d10 Leucine)
Figure 5.2b
D-Leucine internal standard curve
(Internal standard = d10 Leucine)
Figure 5.3
Maternal enrichment (atoms percent excess) by labelled leucine during infusion.
Figure 5.4a
Fractional extraction ratio of L-Leucine in AGA and SGA groups
Figure 5.4b
Fractional extraction ratio of D-Leucine in AGA and SGA groups
Figure 5.5a
Mean umbilical vein (UmV) and umbilical artery (UmA) L-Leucine concentration (Umol/L) in AGA and SGA groups.
Figure 5.5b
Mean umbilical vein (UmV) and artery (UmA) D-leucine concentration (Umol/L) in AGA and SGA groups
Figure 5.6a
Differences between umbilical vein and artery L-Leucine concentration in AGA and SGA groups
Figure 5.6b
Differences between umbilical vein (UmV) and artery (UmA) D-Leucine concentration in AGA and SGA groups.
Figure 5.7a
Feto:maternal (F:M) ratio of L-leucine in AGA and SGA groups
Figure 5.7b
Feto:Maternal (F:M) ratio of D-Leucine in AGA and SGA groups
Figure 5.8
Labelling (atoms percent excess) of umbilical vein (UmV) by deuterium labelled leucine.
Figure 5.9a
Uptake (Umol/kg/min) of L-Leucine by AGA and SGA fetuses
Figure 5.9b
Mean uptake (Umol/kg/min) of D-Leucine by AGA and SGA fetuses.
CHAPTER 6

FETAL OXYGEN CONSUMPTION
6.1 Introduction

6.2 Background to the study

6.3 Subjects and methods

6.4 Results

6.5 Discussion
6.1 Introduction:

Since Huggert demonstrated that oxygen saturation in the umbilical vein of the fetal lamb was lower than that of the mother, various experiments have been conducted both in the acute and in the chronic state in order to understand fetal physiology and behaviour (Dawes, 1994). Despite recent advances, various constraints have restricted most metabolic experiments to animal fetuses which may differ from human fetuses in a large number of aspects of physiology such as feto-placental and uterine circulations and body composition. The main limiting factors have included ethical considerations and technical constraints. As late as 1985, it became possible to obtain blood from the human fetus before delivery thereby enabling aspects of physiology and metabolism to be studied in utero in some pathological conditions (Soothill et al. 1986; Nicolaides et al. 1986 & 1988; Weiner & Williamson, 1989). Despite this advance, most studies of fetal physiology have been based on estimates made on blood samples obtained at delivery (Lin et al. 1980; Nieto et al. 1994). It is therefore not surprising that most of the data on human fetal metabolism are extrapolated from animal studies. The inaccessibility of the human fetal vessels (vein and artery), uterine artery and vein has also been a major constraint on studies of human physiology.

The fetus depends upon the mother for the supply of substrates for its caloric requirements. In human beings, it has been estimated that the caloric requirement of the fetus constitutes less than 10% of the daily caloric requirement of the mother (Bell, 1985). In order to determine the caloric requirements of the preterm viable fetus and therefore postnatal nutritional needs and also to enable comparative physiology with other
animals which may have different fetal growth rates (for example the lamb with a threefold higher growth rate), it is important to study and understand fetal caloric requirements (Sparks et al. 1980).

The effect of some factors such as temperature fluctuations and gravity on fetal caloric requirements are absent in utero. Studying fetal caloric requirements, therefore provides a unique model for understanding the relationship between body size and metabolic rate. Indirect measurements of fetal caloric requirements can be made by determining the oxygen consumption (Sparks et al. 1980) while direct measurements can be made by bomb calorimetry of fetal carcass (Battaglia, 1970; Rattray et al. 1974; Battaglia & Meschia, 1978).

The very nature of intrauterine existence implies an evolutionary series of physiologic mechanisms which provide the fetus with both temporary and permanent functions and, in the final analysis prepare it for extrauterine existence. All previous estimates of fetal oxygen consumption have concluded that it does not change with fetal weight, yet studies of preterm (Sauer et al. 1984) and term (Hill and Rahimtulla, 1965) babies have shown that the preterm neonate's metabolic rate rises with weight.

The energy balance equation:
\[(\text{Energy}_{\text{intake}} = \text{Energy}_{\text{excreted}} + \text{Energy}_{\text{expended}} + \text{Energy}_{\text{components}})\]
outlines the relationship between energy intake and usage. Since the rate of body building increases with gestation, it can be deduced from this equation that energy expended and present in components must increase in the last trimester. Fetal metabolic rate should therefore increase with gestation.
Indirect studies and extrapolations from animals have estimated human fetal oxygen consumption (VO$_2$) to be about 5-8 ml. kg.$^{-1}$ min.$^{-1}$ (Romney et al. 1955, Sauer et al, 1984; Battaglia & Meschia 1986) and therefore a caloric expenditure of 4.9 kcal.$^{-1}$ kg.$^{-1}$ min.$^{-1}$ of oxygen at STP (Standard Temperature and Pressure) (Sparks et al. 1980). Studies of preterm neonates on the other hand have estimated the oxygen consumption rate to be 8.66 ml. kg.$^{-1}$ min.$^{-1}$ (Reichman et al. 1982). This is based on feeding the neonate with milk that provides 115 kcals. kg.$^{-1}$ day.$^{-1}$. Battaglia and Meschia (1978) have stated that the metabolic rate of the mammalian fetus is relatively independent of fetal size. If this is true then it is implied that fetal caloric requirements in utero remain fairly constant, while a rise occurs after delivery - a pattern which does not fit the ontogeny of any other fetal - neonatal physiological process. This chapter describes the in-vivo measurements of human fetal metabolic rate (oxygen consumption) at caesarean section. The aims of the study were to determine the normal range of fetal metabolic rate over a wider weight range and to determine the relationship if any such a metabolic rate has with weight.

6.2 Subjects and Methods:

Forty women undergoing elective caesarean section for various obstetric indications were studied. All were infused with different mixtures of stable isotope labelled D-, and L- amino acids and mannitol for 2 hours before delivery. The blood flow, mannitol and amino acids studies are described in chapters 2,3,4 and 5.

At caesarean section, a loop of the umbilical cord was exteriorized on opening the lower segment of the uterus but before delivering the baby. Umbilical vein blood flow was measured by applying a transonic time
flow probe to the vein as described in Chapter 2. The baby was then
delivered and heparinised blood samples were collected from both the
umbilical vein and the artery.

Fifty microlitres of each blood sample was injected into an OSM\textsuperscript{T}M3
Hemoximeter (Model Number 983-718, Radiometer International) for the
estimation of haemoglobin oxygen saturation (HbO\textsubscript{2}SAT ), total oxygen
content (O\textsubscript{2}ct), haemoglobin (Hb), carboxyhaemoglobin (HbCO),
bicarbonate (HCO\textsubscript{3}) and methaemoglobin (MetHb). An internal correction
factor for fetal haemoglobin (HbF) was programmed for this
measurement. A further 30 microlitres of umbilical vein and artery blood
sample was injected into a 1306 pH/Blood Gas Analyzer (Instrumentation
Laboratory, UK) for PaO\textsubscript{2}, pH, HCO\textsubscript{3} and base excess estimation. Each
measurement in the Hemoximeter and the Blood Gas Analyzer was
performed twice and the coefficient of variation was 1\% for the
Hemoximeter and 0.8 \% for the Gas Analyzer.

Oxygen consumption (VO\textsubscript{2}) was then calculated from the measured
oxygen saturation, haemoglobin concentration and umbilical vein blood
flow using from the following equations (Paulick et al. 1992). The
assumption was made that 1 gram of haemoglobin contained 1.34 ml of
dissolved oxygen (Ganong, 1991).

1. \( O_2 \text{ content (ml/dl)} = 0.0134 \times \text{Hb concentration (g/dl)} \times O_2 \text{ saturation (\%)} \)
2. \( O_2 \text{ delivery (ml/min)} = O_2 \text{ content UmV (ml/dl)} \times \text{UmV blood flow (ml/min)}/100 \)
3. \( VO_2 \text{ (ml/min)} = O_2 \text{ content (UmV-UmA)} \times \text{UmV blood flow (ml/min)}/100 \)
4. \( VO_2 \text{ (ml/kg/min)} = VO_2 \text{ (ml/min)} /\text{Birth weight (kg)} \)
The fractional extraction of oxygen by the fetus was calculated from the following equation:

$$\text{Fractional extraction (\%)} = \frac{\text{UmV HbO}_2\text{SAT} - \text{UmAHbO}_2\text{SAT} \times 100}{\text{UmV HbO}_2\text{SAT}}$$

where

UmV = Umbilical vein
UmA = Umbilical artery
HbO$_2$SAT = haemoglobin oxygen saturation.

6.3 Results:

Of the 40 babies, 16 were small for gestational age (SGA) while 24 were appropriate for gestational age (AGA). The gestational ages at delivery in the SGA group varied from 29 to 39 weeks with a mean of 36.3 (SD, 3.12) weeks while that in the AGA group varied from 33 to 41 weeks with a mean gestation of 38.2 weeks (SD, 1.69). The mean birth weight was 3250 g (SD, 550) in the AGA group and 2039 g (SD, 580) in the SGA group (Table 6.1). Figure 6.1 shows the relationship between birth weight and gestational age at delivery. This relationship is linear with a correlation coefficient of 0.79 (P < 0.05).

Table 6.2 shows the mean haemoglobin and blood gases in the two groups. The mean haemoglobin oxygen saturation (HbO$_2$SAT) in the umbilical vein of the 40 babies was 61.8 % (SD, 17.1). The mean HbO$_2$SAT in the appropriate for gestational age group (65.0 %; range 42.9% to 84.1%) was significantly higher (p < 0.05) than that in the small for gestational age group (51.25%; range 15.9% to 78.1%). The total oxygen content in the SGA group was significantly lower (P < 0.05) than that in the AGA group.
The bicarbonate level in SGA group was higher than in the AGA group but this was not statistically significant.

The mean oxygen consumption (VO$_2$) in the 40 fetuses was 6.6 ± 2.0 ml. kg.$^{-1}$ min.$^{-1}$. The mean VO$_2$ in the AGA group was 8.4 ± 2.1 ml. kg.$^{-1}$ min.$^{-1}$ compared to 6.4 ± 2.6 ml. kg.$^{-1}$ min.$^{-1}$ in the SGA group. The relationship between oxygen consumption per kg of baby and birth weight is shown in Figure 6.2 (r = 0.38, p < 0.05). Figure 6.3 shows that the relationship between total oxygen consumption and birth weight is statistically significant (r = 0.8, p < 0.001). This relationship is exponential with a steep rise between birth weight 1800g and 4000g. A logarithmic plot of this relationship is shown in Figure 6.4. The relationship between whole body VO$_2$ and birth weight (M) can be described by a power function with an exponent significantly greater than unity (ie VO$_2$ (ml. min. $^{-1}$) = 3.67 M$^{1.6}$ (kg) (Figure 6.5). Further, the weight specific oxygen consumption (ie expressed per kilogram of birth weight) also showed a power relationship with body mass (VO$_2$ (ml.kg.$^{-1}$min.$^{-1}$) = 4.17M$^{0.53}$(kg) (Figure 6.6). There is a doubling in energy expenditure of the fetus per kilogram body weight as the body weight increases from 1000g to 3500g. Body mass was the best predictor of oxygen consumption; a single relationship best described the relationship between the two variables and gender and gestational age. There was no significant (r = 0.23, p > 0.05) correlation between VO$_2$ and gestational age(Figure 6.7).

Figure 6.8 shows that there was no significant relationship between blood flow and oxygen consumption. There was also no significant relationship between umbilical vein haemoglobin concentration and birth weight (Figure 6.9). There was, however, a linear relationship between umbilical
vein oxygen saturation and birth weight (Figure 6.10). The correlation coefficient for this relationship was 0.4 (P < 0.05).

Figure 6.11 shows the relationship between the relative oxygen extraction by the fetus and oxygen consumption. This relationship is linear with a correlation coefficient of 0.75 (P < 0.05). The association between oxygen consumption and placental weight is shown in Figure 6.12. This figure shows that there is no relationship between oxygen consumption and placental weight.

The relationship between core body temperature and oxygen consumption was studied in nine of the neonates by measuring their rectal temperatures immediately after delivery. This relationship is shown in Figure 6.13. Core body temperature had no effect on oxygen consumption.

6.4 Discussion

The mean umbilical vein and artery haemoglobin oxygen saturation, pH, base excess, bicarbonate concentration, and carboxyhaemoglobin are similar to those previously reported in unstressed fetuses at mid gestation (Soothill et al. 1986; Bozzetti et al. 1987) and term fetuses in early labour (Nieto et al. 1994). The difference between the haemoglobin concentration in the two groups was also not statistically significant even though growth retarded babies are more likely to be polycythaemic (Merberg, 1980).

The insignificant difference in the pH of the umbilical vein and artery samples in the small and appropriate for gestational age groups confirm the observations of Lin et al. (1980) who also stated that most differences in pH in the two groups occur because the small for gestational age fetuses
tolerate labour poorly. However, Soothill et al. (1987) have shown that in severely growth retarded fetuses, alteration in the acid base status with a tendency towards acidosis occurs even before labour. In fact 5 fetuses in this study with birth weights below the 3rd centile had a mean pH (7.22) that was significantly lower than in the appropriate for gestational age fetuses (7.34). The lack of a significant difference in the pH and base excess in these groups may be due to the fact that, not all the fetuses in the small for gestational age group were in fact growth retarded.

The oxygen consumption rate in the human fetus in this study varied from 2.4 - 12.9 ml. kg\(^{-1}\) min\(^{-1}\) and the value of 5 - 8 ml. kg\(^{-1}\) min\(^{-1}\) reported by Battaglia & Meschia (1986) falls within this range. However, the oxygen consumption rates from which Battaglia & Meschia obtained their mean values were from animals such as the sheep. In addition, the two studies that were performed on human fetuses, were mainly in normal size fetuses (Romney et al. 1955) or as in another study, only in one fetus (Sandiford & Wheeler, 1924). The birth weights in the present study varied from 840 g to 4360 g while in the previous most extensive human study of 6 fetuses their weights varied only between 2900 and 3200g (Romney et al. 1955). In addition, measurements from the study by Romney et al. (1955) were indirect - calculated by subtracting the metabolic rate of the uterus and placenta from that of the uterus and its contents (determined by subtracting the metabolic rate of a non-pregnant woman from that of a pregnant woman). The mean oxygen consumption (6.6 ml. kg\(^{-1}\) min\(^{-1}\)) in the present study was, however, within Battaglia & Meschia (1986) range of 5-8 ml. kg\(^{-1}\) min\(^{-1}\) but higher than the value of 4.8ml. kg\(^{-1}\) min\(^{-1}\) in normal term infants up to 6 hours after delivery (Hill and Rahimtulla, 1965).
Various factors could be responsible for the wide variation in oxygen consumption observed in the human fetus. In order to determine the effect of some of these factors on oxygen consumption, their correlation coefficients with oxygen consumption (or determinants of oxygen consumption) were calculated. Since birth weight increases with gestation, the first objective was to determine the effect of gestational age on oxygen consumption. As shown in figure 6.7, the correlation coefficient for this relationship was 0.23 (P > 0.05). The association between oxygen consumption and gestational age was therefore only small. However, there was a linear correlation between blood flow and total oxygen consumption (ml/min). These findings are similar to those of Dawes et al (1959). In fact, Paulick et al. (1992) observed that when the umbilical vein was gradually occluded (thereby reducing blood flow), oxygen consumption in the lamb decreased exponentially. Since the mean umbilical blood flow in the small for gestational age fetuses is lower than in the appropriate for gestational age fetuses (Chapter 2), the observation of a lower oxygen consumption rate in small for gestational age fetuses is therefore not surprising.

Another variable that was used in calculating oxygen consumption was umbilical vein haemoglobin. As shown in Table 6.2, there was no statistically significant difference between the haemoglobin concentrations in the SGA and AGA groups. Similarly, there was no significant association between umbilical venous haemoglobin concentration and fetal oxygen consumption (Figure 6.9). Another possibility which might explain the apparent rise in weight specific oxygen consumption is that the smaller babies lost more heat once the uterus was opened and the cooling effect decreased their oxygen consumption. To study this, oxygen consumption was measured in 9 babies weighing between 1800g and 3000 g
in which central body temperature was also measured: the range of body temperatures was small (suggesting that cooling was not significant) and there was no relationship between oxygen consumption and body temperature over this range. Hill and Rahimtulla (1965) also concluded from their observations that ambient temperature did not adversely affect oxygen consumption of the neonate.

All the factors discussed above apply to all animals and therefore ought to affect animal fetal oxygen consumption in a similar manner. Why, therefore is there a wider variation in human fetal oxygen consumption with birth weight? From the energy balance equation (page 192), it is known that the fetus expends energy in storing fat, protein and other components of the body. The human fetus is unusual among mammalian fetuses in showing a high rate of fat deposition during intrauterine growth (Heim et al. 1981; Battaglia & Meschia, 1986). Of those animals in which the oxygen consumption per kilogram of the fetus has been measured it falls close to 7 ml. kg\textsuperscript{-1} min\textsuperscript{-1} at term (Battaglia & Meschia, 1986) whereas in this study, it rose up to 12.9 ml. kg\textsuperscript{-1} min\textsuperscript{-1} in the term fetus. In most animals the newborns are much leaner than the human newborn baby in which fat typically constitutes 15% of body weight at term (Heim et al. 1981; Battaglia & Meschia, 1986). In the human fetus, the rate of protein accretion is almost constant (2.5 to 5.3 g/kg/day) from 26 to 40 weeks gestation (Mettau et al. 1977). However, the rate of fat accretion rises from 0.7 g/kg/day between 26 and 31 weeks to 8.3 g/kg/day between 35 and 38 weeks and to about 14.3 g/kg/day at 40 weeks. In pigs, sheep and horses, there is very little subcutaneous fat at birth (Widdowson & Dickerson, 1964) and therefore little is stored during gestation. In small for gestational age fetuses, the fat content is smaller compared to that in appropriate for gestational age fetuses (Widdowson & Dickerson, 1964). The rate of fat gain
is also much slower. A remarkable feature of the growth of the human baby during intrauterine life is that despite differences in rates of growth between babies (e.g. between large, appropriate and small for gestational age fetuses) the amount of body weight due to fat in relation to body mass conforms closely to a single power relationship (Heim et al., 1981) with an exponent of 2.1. In other words, as human fetal body mass increases, fat mass increases disproportionately; in fact, fat mass appears to grow at about 7.14 % per day on average. Over the same period the non-fat dry body mass increases virtually linearly and also the hydration of the tissues decreases from about 90 % to 70 %, developmental features shared with other mammals (Battaglia & Meschia, 1986). Since the transfer of lipid metabolites across the placenta is limited, the fetus must make body fat from glucose and amino acids, the major energy containing substrates transferred across the placenta (Battaglia & Meschia, 1986).

It can therefore be inferred that the observed paradoxical increase in weight specific oxygen consumption might be due mainly to the energy costs of synthesizing fat from glucose and/or amino acids although a small proportion might be due to the loss of water per weight of metabolically active tissue and increase of less metabolically active tissue such as muscle and bone. Body fat accretion in the fetus appears to accelerate rapidly from about 28 weeks of age when the fetal weight is about 1 kg and the body fat constitutes about 2 % of the body weight (Heim et al., 1981). This proportion is close to that observed in lean tissues such as kidney and muscle and is probably due to irreducible contribution of functionally important lipid-containing structures such as mitochondria and cell membranes. It should, therefore, be possible to test the hypothesis that the increase in the weight specific oxygen consumption with fetal body weight is due to the energy cost of lipid synthesis and storage by determining
whether the extra consumption of energy in fetuses above 1 kg in body mass can be accounted for by the known (Heim et al., 1981; Sparks, 1980) rate of fat synthesis.

This was done by assuming that in all of the babies whose oxygen consumption was measured, the percentage fat at a given body weight could be predicted from the power relationship described above and that the fractional rate of fat accretion was 7.14 % per day. The energy cost for fat synthesis from glucose was taken as 8 kJ/g (Sauer, 1986), from amino acids as 10 kJ/g (Sauer et al., 1979) and the mix of glucose and amino acids taken up by the fetus was 50:50, as in the lamb (Battaglia & Meschia, 1986). Thus, the assumed value was 9 kJ/g fat. The oxygen consumption per g of non-fat tissue is more or less constant throughout gestation because although the degree of tissue hydration falls, the proportion of highly metabolic tissue (eg liver, kidney etc.) falls as the musculature and skeleton grow; the effects of these opposing influences roughly cancelling each other (Bell et al. 1985). The constant value of 4.17 ml/kg lean tissue has therefore been used, the value obtained by extrapolation of the observed relationship of VO₂ and body weight to 750 g.

The results of these calculations (Table 6.3) show that almost all the differences between the oxygen consumption predicted for lean tissue containing 5 % fat and 90 % water (4.2 ml. kg⁻¹ min⁻¹) in a fetus of 1 kg and the observed weight specific oxygen consumption for fetuses of up to 4 kg birth weights may be accounted for by the energy cost of fat synthesis from non-fat sources.

It may be, therefore, that in the developing human fetus, most energy expending processes such as the circulation of the blood or protein
synthesis in lean tissues, occur at a rate which is linearly related to body weight but that the synthesis of body fat from carbohydrate and amino acids imposes a greater energetic demand upon placental delivery of oxygen and energy supplying substrates than is observed in other animals. Chien et al. (1993) have shown that the safety margin for the transfer of amino acids such as leucine and phenylalanine across the human placenta is small and in addition there is preliminary evidence that the transfer of leucine occurs at a slower rate in SGA than AGA fetuses (Konje et al., 1995). The present results indicate that oxygen consumption in SGA babies is also diminished at term. Thus any limitations of maternal delivery or placental transfer of oxygen and substrates are more likely to result in growth retardation in the human fetus than in the fetuses of other mammals.

It is reasonable therefore to suggest that the wide variation in oxygen consumption observed in this study is a reflection of the energy expended in fat accumulation; the very small fetuses spending very little energy in fat storage compared to the larger ones. The sudden rise in $V_{O_2}$ after birth weight 1800 g will be in keeping with this observation. Since fetal weight gain is a function of gestational age in the appropriate for gestational age group, these findings suggest that, the maturational processes occurring in various organs and physiological systems also occur in the metabolic rate. This rate increase is rapid during the period of rapid fetal growth. The concept of a constant metabolic rate in mammalian fetuses as stated by Battaglia and Meschia (1978) therefore has to be challenged. It may be true in other mammals such as sheep and horses but does not appear to be the case in human fetuses. However, it is important to acknowledge the fact that methodological limitations of this work could have contributed to some of the variations observed. Since oxygen consumption was
determined from variables such as blood flow, haemoglobin concentration, haemoglobin oxygen saturation and birth weight, any errors in these measurements would certainly be reflect in the values obtained.

During the neonatal period feeding of preterm infants, is geared towards providing enough calories (irrespective of the size at birth and whether the neonate is small or appropriate for gestational age). The findings in this study suggest that it may be possible to tailor the caloric requirements of a particular fetus based on its birth weight (compared to the expected weight) and also on its projected weight at the end of a specific time period. The allometric equation defining birth weight and oxygen consumption can be used to calculate the caloric requirements of a preterm neonate very accurately.
Table 6.1  Mean ± SD gestational age, birth weight, birth weight centiles, umbilical vein blood flow and oxygen consumption in AGA and SGA groups

<table>
<thead>
<tr>
<th></th>
<th>AGA (n = 24)</th>
<th>SGA (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean gestational age (weeks)</td>
<td>38.2 ± 1.7</td>
<td>36.3 ± 3.1</td>
</tr>
<tr>
<td>Mean birth weight (grams)</td>
<td>3250 ± 550</td>
<td>2039 ± 580</td>
</tr>
<tr>
<td>Birth weight centiles (range)</td>
<td>18 - 105</td>
<td>0.5 - 8</td>
</tr>
<tr>
<td>Mean blood flow (ml/kg/min)</td>
<td>88.4 ± 6.5</td>
<td>61.0 ± 6.2</td>
</tr>
<tr>
<td>Mean oxygen consumption (ml/kg/min)</td>
<td>8.4 ± 2.1</td>
<td>6.4 ± 2.6</td>
</tr>
<tr>
<td>Gorup</td>
<td>AGA (N = 24)</td>
<td>SGA (N = 16)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Haemoglobin (Hb) g/dl</td>
<td>15.8 ± 1.71</td>
<td>15.75 ± 1.79</td>
</tr>
<tr>
<td>Haemoglobin oxygen saturation (%)</td>
<td>65.0 (42.9 - 84.1)</td>
<td>51.25 (15.9 - 78.1)</td>
</tr>
<tr>
<td>Oxygen content (ml/dl)</td>
<td>14.16 ± 2.44</td>
<td>12.32 ± 3.88</td>
</tr>
<tr>
<td>pH</td>
<td>7.34 ± 0.07</td>
<td>7.32 ± 0.02</td>
</tr>
<tr>
<td>PaO₂</td>
<td>4.05 ± 0.79</td>
<td>3.17 ± 0.94</td>
</tr>
<tr>
<td>HCO₃</td>
<td>22.85 ± 1.78</td>
<td>24.47 ± 1.38</td>
</tr>
</tbody>
</table>
Table 6.3:
Relationship between body weight, fat mass, fat synthesis and extra oxygen consumption (VO2) in the human fetus.

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Fat mass (g)</th>
<th>Fat synthesis (g)</th>
<th>VO2 due to fat synthesis (ml/kg/min)</th>
<th>Total calculated VO2 (ml/kg/min)</th>
<th>Observed VO2 (ml/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500</td>
<td>70</td>
<td>5.0</td>
<td>1.04</td>
<td>5.21</td>
<td>5.18</td>
</tr>
<tr>
<td>2000</td>
<td>140</td>
<td>10.0</td>
<td>1.56</td>
<td>5.7</td>
<td>6.03</td>
</tr>
<tr>
<td>2500</td>
<td>225</td>
<td>16.0</td>
<td>2.01</td>
<td>6.10</td>
<td>6.79</td>
</tr>
<tr>
<td>3000</td>
<td>355</td>
<td>25.3</td>
<td>2.64</td>
<td>6.81</td>
<td>7.48</td>
</tr>
<tr>
<td>3500</td>
<td>523</td>
<td>37.3</td>
<td>3.33</td>
<td>7.5</td>
<td>8.11</td>
</tr>
<tr>
<td>4000</td>
<td>731</td>
<td>52.2</td>
<td>4.07</td>
<td>8.24</td>
<td>8.71</td>
</tr>
</tbody>
</table>

a From Ziegler et al. 1976

b Assumed to be an average of 7.14% per day (Heim et al. 1981)

c Energy cost of fat synthesis from glucose and amino acids assumed to be 9kJ/g and the energy equivalent of oxygen as 20kJ/g.

d Calculated as the oxygen consumption to fat synthesis plus 4.17 ml/kg/min.

e From data in Figure 6.2.
Figure 6.1
The relationship between birth weight and gestational age

\[ y = 30.933 \times 10^{2.9124e-5x} \quad R^2 = 0.615 \]
Oxygen consumption (ml/kg/min)

\[ y = 4.1747 \times 10^{7.9103e-5x} \]

\[ R^2 = 0.144 \]

Figure 6.2
Oxygen consumption versus birth weight
Figure 6.3
The relationship between total oxygen consumption and birth weight

\[ y = 3.5197 \cdot 10^{(2.6116e-4x)} \quad R^2 = 0.654 \]
Figure 6.4
The relationship between log oxygen consumption and log birth weight

\[ y = 3.2022 \times 10^{(6.1635e-2x)} \quad R^2 = 0.617 \]
Figure 6.5
Fetal oxygen consumption as a function of birth weight

\[ y = 8.1650 + 5.9334e^{-4}x \quad R^2 = 0.649 \]
Figure 6.6
Total fetal oxygen consumption as a function of birth weight

\[ y = 7.8470 \times 10^{(1.5096e-4x)} \]

\[ R^2 = 0.267 \]
Figure 6.7
The relationship between oxygen consumption and gestational age

\[ y = -1.6018 + 0.24104x \quad R^2 = 0.059 \]
Oxygen consumption versus umbilical vein blood flow

\[ y = 6.0380 \times 10^{(7.2715e-4x)} \]

\[ R^2 = 0.011 \]

Figure 6.8
Oxygen consumption versus umbilical vein blood flow
Umbilical vein haemoglobin (g/dl)

$y = 16.222 - 1.8668 \times 10^{-4}x$

$R^2 = 0.007$

Figure 6.9
The relationship between umbilical vein Hb and birth weight
Figure 6.10
Birth weight versus umbilical vein oxygen saturation

\[ y = 36.289 \times 10^{(7.5613 \times 10^{-5} \times x)} \quad R^2 = 0.184 \]
Figure 6.11
The relationship between oxygen extraction and oxygen consumption
Figure 6.12
The relationship between placental weight and oxygen consumption

\[ y = 6.5515 + 1.8347e^{-3x} \quad R^2 = 0.010 \]
Figure 6.13
The relationship between core body temperature and oxygen consumption

\[ y = 36.296 + 7.9513 \times 10^{-5} x \quad R^2 = 0.011 \]

\[ y = 9.0187 \times 10^{-2} + 3.1752 \times 10^{-3} x \quad R^2 = 0.445 \]
Conclusions

The following conclusions can be made from the results of these studies.

- The transit-time flowmetry technique can be successfully applied to the measurement of umbilical vein blood flow at Caesarean section.
- Mannitol transfer across the placenta is carrier mediated.
- Fetal oxygen consumption rises exponentially with weight.
- Findings of diminished concentration of leucine and glycine in the plasma of small for gestational age fetuses have confirmed previous observations.
- Leucine and glycine transfer across the placenta is defective in SGA pregnancies.
- D-leucine is transported actively across the placenta.
- The transporter for D-leucine may be similar to that for L-leucine.
- Total fetal leucine and glycine uptake is markedly reduced in SGA fetuses.
- Utilisation of glycine is slower in SGA neonates during the first hour of life.
Scope for Future Research

The results from these studies suggest that decreased delivery of amino acids to the fetus may contribute towards diminished protein and fat accretion and subsequently thus lower growth rates in utero. In addition, the observed decrease in the clearance rate of mannitol across the placenta of small for gestational age pregnancies would support the hypothesis that placental function in terms of its transport capacity is compromised in these pregnancies. The transport capacity of the placenta is dependent upon many factors including:

1. surface area of exchange (various elements of villi and trophoblasts) and
2. concentration and function of transporters for nutrients.

Therefore future work in this area should be directed towards the potential contribution of these factors to the compromised transport capacity of the placenta.

- **Morphology of surface area of exchange:**
  
  In an attempt to understand why there should be variations in amino acid concentration and transport in SGA and AGA fetuses, the relationship between placental structure and amino acid transport should be examined. This will examine the relationship between any diminution in placental exchange surface area and decreased transport function. The placental exchange surface area could be examined using stereological techniques and scanning electron microscopy.

- **Quality of exchange:**
  
  Diminished transfer of amino acids across the placenta could be due to inadequate production of transporters or transporter dysfunction. Since the expression of various transporters on the placenta can now be studied using molecular biology techniques, a quantitative study of mRNA expression and the total protein for leucine and glycine transporters would investigate whether there is decreased production of these transporters. Mutation of the transporters could be explored by amino acids sequencing.
Another interesting observation in this study was a higher glycine concentration in SGA neonates at one hour of life. This could suggest that the metabolism of glycine is diminished in SGA neonates. However, it is possible that SGA fetuses through an intrauterine adaptive mechanism have already "switched on" their gluconeogenic pathway and the alternate metabolic pathway involving interconversion to serine to their maximal potential, and this continues after birth. In contrast, the AGA fetuses may only "switch on" this pathway to maximum after birth.

- **Neonatal metabolism:**
  
  In future studies, this hypothesis could be tested by using stable isotope labelling at a position on glycine where the isotope remains in the metabolite, providing an opportunity for the determination of how much interconversion occurs before and after delivery in both the SGA and AGA fetuses.
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