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High-performance liquid chromatographic measurement of leucine and α-ketoisocaproate in whole blood: application to fetal protein metabolism

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ABSTRACT

Evaluation of fetal protein metabolism requires measurement of a number of variables including umbilical blood flow, CO_2 radioactivity, as well as plasma specific activities, whole blood concentration, and radioactivity of leucine and α-ketoisocaproate. This report details methods of analysis for whole blood concentration and radioactivity of leucine and α-ketoisocaproate using high-performance liquid chromatography that can be done on minimal blood volumes and are sufficiently accurate to detect the small arteriovenous differences important in measurements of fetal metabolism. Using these methods, the important components of fetal protein metabolism such as protein synthesis can be calculated with sufficient accuracy to detect differences as small as 10% provided appropriate experimental designs are used.

INTRODUCTION

Fetal life is characterized by brisk growth and rapid remodelling of tissues. The vigorous turnover of protein (*i.e.* continuous protein synthesis and breakdown) that occurs in fetal life [1] is in accord with the need for tissue remodelling. Protein accretion (an important component of growth) results when protein synthesis is greater than breakdown. In spite of the careful balance that must be maintained between protein synthesis and breakdown to achieve normal fetal growth rates, little is known about the control of fetal protein metabolism. Measurements of fetal protein synthesis, breakdown and accretion have been hampered not only by the technical difficulties of accurately measuring protein synthesis [2,3], but also by cumbersome methods of measuring protein accretion [4].

Whole body protein synthesis (*i.e.* non-oxidative amino acid disposal) has been measured in adults by a number of tracer methods [2,5]. Infusion of radioactive leucine until intracellular specific activity, as measured by its transamination product, α-ketoisocaproate, becomes constant, allows leucine disposal (or turnover) to be calculated as tracer infusion rate divided by plasma α-ketoisocaproate specific activity [6,7]. In fetal life, however, net loss of tracer (either as leucine or as α-ketoisocaproate) across the umbilical circulation (to the placenta and mother) represents an additional loss of leucine not present in adults [3,8] that must be measured before fetal leucine disposal can be quantified. Non-oxidative leucine disposal (presumably for protein synthesis) can then be calculated as the difference between total leucine turnover and the rate of leu-

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cine decarboxylation, a measurable quantity. In addition, fetal leucine uptake across the placenta (whether as leucine itself or as its α-keto analogue, α-ketoisocaproate) is used either for fetal protein accretion or decarboxylation. Because decarboxylation can be measured, measurement of such uptake allows calculation of fetal accretion of leucine into protein.

Although methods appropriate for measurement of many of the variables needed to describe fetal protein metabolism have been reported [8–13], we were unable to find previously published methods for analysis of whole blood concentrations of leucine and α-ketoisocaproate that were sufficiently accurate to measure small umbilical venoarterial differences and that we could perform on blood volumes small enough that repetitive sampling will not excessively diminish fetal blood volume. The purpose of this report is to describe such methods and apply them to the calculation of fetal protein metabolism to show that they are suitable for such measurements.

EXPERIMENTAL

Animals

Four pregnant ewes with gestational ages between 115 and 117 days (Torrell Ranch, Ukiah, CA, USA) were used for this experiment. On the day of surgery, the animals were premedicated with ketamine, intubated, and placed under general anesthesia (*ca.* 2% isofluorane). The midline of the maternal abdomen was incised and a hysterotomy performed over the area of the fetal abdomen. The fetal skin was incised *ca.* 2–3 mm from the umbilical ring and a catheter placed in the umbilical vein. This incision was then closed and the uterus reincised over the fetal hindlimbs. One fetal hindlimb was withdrawn through the incision, the skin incised, catheters placed in a hindlimb artery and vein, then advanced to the abdominal aorta and inferior vena cava, respectively. An additional venous catheter was placed in the other fetal hindlimb by similar methods. The hysterotomy and maternal abdominal incisions were closed, and the catheters tunnelled to the maternal flank where they were stored in a pouch until needed.

Prior to operation and for three days postoperatively, each animal received antibiotics (2.5 ml of Di-Trim 48% injection containing 80 mg/ml trimethoprim and 400 mg/ml sulfadiazine, Syntex Animal Health, West Des Moines, IA, USA). We allowed six days for the animal to recover before any experiments were begun. Catheter patency was ensured by filling the catheters with saline (0.9%) containing heparin (100 U/ml) every other day. The animals had free access to food and water during this recovery period. On completion of the study protocols outlined below, the ewe and fetus were killed by barbiturate overdose (Beuthanasia-D Special, Schering, Kenilworth, NJ, USA). The fetus was then weighed and all catheter positions confirmed.

Experimental design

We performed three sets of measurements in each of the four animals to separate the contributions of inter- and intra-animal variability to the total variability. Experiments were initiated by beginning an infusion of [1-^{14}C]leucine (Du Pont NEN Research Products, Boston, MA, USA) in sterile saline into the fetal inferior vena cava catheter at $5.5 \cdot 10^6$ dpm/min. Two hours later, an infusion of antipyrine in sterile saline (0.9%) was begun at 5 mg/min (*ca.* 2–2.5 mg/kg per min), also into the fetal inferior vena cava. Both infusions were continued for an additional hour, then three sets of blood samples were drawn at 15-min intervals. Blood drawn simultaneously from the fetal umbilical vein and aorta was analyzed for whole blood contents of antipyrine, leucine, and α-ketoisocaproate, as well as for radioactivity as $^{14}CO_2$, [^{14}C]leucine, and [^{14}C]α-ketoisocaproate. In addition, arterial plasma was analyzed for leucine and α-ketoisocaproate specific activities. Arterial blood was also analyzed for hematocrit, blood gases, and pH. The total amount of blood removed for these analyses totalled 9.3 ml, less than 3% of the fetal blood volume (*ca.* 350 ml based on the weight measured at autopsy). The above protocol was repeated twice more at 48-h intervals in each sheep.

Reagents and apparatus

Antipyrine, phenacetin, sodium phosphate,

leucine, norleucine, triethylamine, sulfosalicylic acid, sodium acetate, α-ketoisocaproic acid, α-ketocaproic acid, *o*-phenylenediamine, benzethonium hydroxide, and 2-mercaptoethanol were all obtained from Sigma (St. Louis, MO, USA). Phenyl isothiocyanate was protein sequencing grade (Sigma). Dichloromethane, *n*-pentane, acetonitrile, and ethanol were all HPLC grade (E. Merck, Darmstadt, Germany). Scintillation fluid was Ultima Gold LSC (Packard Instrument, Downers Grove, IL, USA).

The chromatographic system for analysis of leucine and α-ketoisocaproate consisted of columns as detailed for each assay below, two Model 110B pumps and a Model 421A system controller (Beckman Instruments, Fullerton, CA, USA), a Model AS2000 autosampler (Hitachi Instruments, San Jose, CA, USA), a Model 2050 variable-wavelength detector (Varian Instruments, San Fernando, CA, USA), a Model 3390A integrator (Hewlett-Packard, Palo Alto, CA, USA), and a Retriever II fraction collector with Model 3100 fraction programmer (ISCO, Lincoln, NE, USA).

The chromatographic system for antipyrine analysis consisted of the column described below, a Model 112 pump, Model 165 variable-wavelength detector, and a Model 504 autosampler (Beckman), and a Model 3390A integrator (Hewlett-Packard).

Assay 1: leucine concentration and radioactivity

Blood or plasma leucine concentration was measured after precolumn formation of a phenylthiocarbamyl derivative [14]. Briefly, 0.5 ml of whole blood or plasma was combined with an equal volume of 6% sulfosalicylic acid containing 200 μ*M* norleucine and 50 μ*M* α-ketocaproic acid (used as internal standards for leucine and α-ketoisocaproate analyses, respectively). Following centrifugation, the supernatant was placed on a cationic exchange column (Poly-Prep, 2 ml bed volume, AG 50W-X8, Bio-Rad Labs., Richmond, CA, USA), and washed four times with 1.0 ml of 0.01 *M* HCl. This eluate was collected and saved for analysis of α-ketoisocaproate (detailed below). The column was then washed three times with 1.0 ml of 4 *M* ammonium hydroxide, and the washings were taken to dryness. The residue was then dissolved in 200 μl of a 7:1:1:1 (v/v) mixture of ethanol, triethylamine, phenyl isothiocyanate, and water, then incubated at room temperature for 20 min, and taken again to dryness.

The dried residue was reconstituted in mobile phase A (0.14 *M* sodium acetate, pH 6.35) and injected onto an HPLC column (150 mm × 3.9 mm I.D., Resolve C_{18}, 5 μm particle size, column temperature 42°C, Waters, Milford, MA, USA). This column was eluted with a linear gradient from 10 to 50% mobile phase B (6:4 acetonitrile–water) over 18 min at 1 ml/min. The leucine eluate peak (detected by UV absorbance at 276 nm) was collected and counted for radioactivity (Packard Tri-Carb scintillation counter, Model B2450, Packard Instrument) using internal standards to correct for quenching. Blood leucine concentration was calculated by the method of peak-height ratio to the internal standard, norleucine (Fig. 1).

The amount of leucine placed on the above column was determined by comparison of the peak height of the unknown to a standard curve (*i.e.* peak-height ratio = 0.0471 × [leucine]; r = 0.9998) generated by subjecting known amounts of leucine to the derivatization and analysis detailed above. The leucine assays done in this manner had an intra-assay coefficient of variation (C.V.) of 1.0% and an inter-assay C.V. of 6.5%.

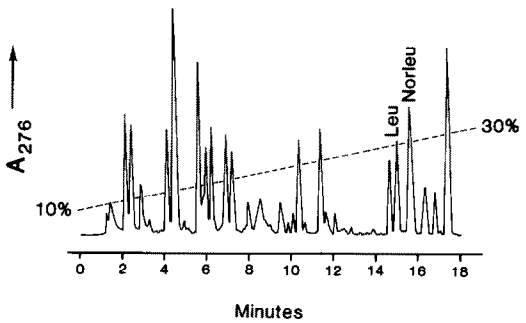

Fig. 1. Chromatogram of whole blood showing the leucine (Leu) peak and the norleucine (Norleu) internal standard peak. The dashed line shows the elution gradient (see Experimental).

The concentration of [^{14}C]leucine in the original whole blood sample (dpm/ml) was calculated as the radioactivity of the column eluate (dpm) divided by the amount of leucine in the column eluate (nmol) to give the specific activity of the column eluate; this latter quantity was then multiplied by the concentration of leucine in whole blood. This determination of the amount of leucine is predicated on the assumption that the process of derivatization is acceptably quantitative. For example, if half of the leucine is lost during derivatization, the leucine concentration will not be affected because the standard curve will loose similar amounts of leucine. However, the recovery of radioactivity from the column will be halved, resulting in a specific activity half of the true value. To test this assumption, we subjected 2.17 ± 0.01 · 10^6 dpm of [1-^{14}C]leucine to derivatization, then injected the sample quantitatively onto the above column. Radioactive recovery was 89.2 ± 0.6% (mean ± S.D., n = 6), indicating excellent recovery of leucine through the derivatization and chromatography processes.

Assay 2: ketoisocaproate concentration and radioactivity

The α-ketoisocaproate content of the ion-exchange eluate (from leucine analysis above) was analyzed by modifying the method of Smeaton *et al.* [10]. A 1-ml volume of derivatization reagent (40 mg of *o*-phenylenediamine in 20 ml of 2 *M* HCl and 100 μl of 2-mercaptoethanol) was added to the 4.0-ml eluate. The solution was heated in the dark under nitrogen (80°C for 2 h). The resulting quinoxalinols were then extracted twice into 2.5 ml of 1:1 (v/v) dichloromethane–*n*-hexane. The organic phase was taken to dryness, reconstituted in 200 μl of methanol, then 200 μl of water, and injected onto the HPLC column (250 mm × 4.6 mm I.D., Ultrasphere ODS, 5 μm particle size, 42°C, Beckman). The elution was carried out by a linear gradient from 80% A (1:12:7 acetonitrile–0.1 *M* NaH_2PO_4, pH 7–water) to 80% B (3:1 acetonitrile–water) over 60 min at 1.0 ml/min. The α-ketoisocaproate peak, detected by UV absorbance (340 nm), was collected and its radioactivity measured (Packard Tri-Carb scintillation counter) using internal standards to correct for quenching. Blood α-ketoisocaproate concentration was determined by comparing the peak height of the α-ketoisocaproate peak to that of the known internal standard (α-ketocaproic acid) (Fig. 2).

The amount of α-ketoisocaproate in the HPLC eluate was determined from a standard curve (*i.e.* peak-height ratio = 0.0285 × [α-ketoisocaproate]; r = 0.9994) relating known amounts of α-ketoisocaproate to α-ketocaproate peak height. This assay was found to have an intra-assay C.V. of 2.2% and an inter-assay C.V. of 5.7%. The radioactivity of α-ketoisocaproate in the original whole blood sample (dpm/ml) was calculated as the radioactivity of the column eluate (dpm) divided by the amount of α-ketoisocaproate in the column eluate (nmol) to give the specific activity of the column eluate; this latter quantity was then multiplied by the concentration of α-ketoisocaproate in whole blood as measured above. As was the case for measurement of leucine specific activity, this measurement of α-ketoisocaproate specific activity assumes that a significant amount of the keto acid is not lost during the derivatization, extraction, and chromatography procedures. To test this possibility, 7.18 ± 0.03 · 10^5 dpm of [^{14}C]α-ketoisocaproate were derivatized as described above. Following derivatization and extraction, 98.2 ± 0.2% of the radioactivity remained in the organic phase. Of this

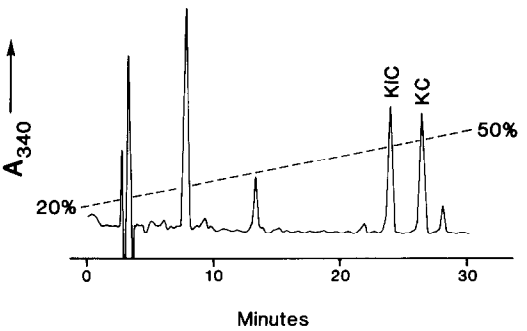

Fig. 2. Chromatogram of whole blood showing the α-ketoisocaproate (KIC) peak and the α-ketocaproate (KC) internal standard peak. The dashed line shows the elution gradient (see Experimental).

98.2%, 93.7 ± 1.0% was collected in the keto acid peak following chromatography, again indicating acceptably complete recovery of α-keto-isocaproate through the isolation procedure.

Assay 3: Blood CO_2 radioactivity

Blood $^{14}CO_2$ was measured by a modification of the method of Hinks *et al.* [12]. A 0.3-ml aliquot of benzethonium hydroxide was placed in a small cup sealed in a vial containing 1.0 ml of 1 *M* HCl. A 0.2-ml aliquot of anaerobically obtained blood was injected into the acidic solution and the vials kept at room temperature for at least 1 h. The cup containing the benzethonium hydroxide (and trapped $^{14}CO_2$) was removed from the vial, the contents were rinsed into scintillation fluid and the radioactivity was measured. Recovery of radioactivity using this method was 99 ± 7%. Counting efficiency was measured using internal standards.

Assay 4: Antipyrine

Whole blood antipyrine concentration was measured using a modification of the method of Teunissen *et al.* [15]. A sample of whole blood (0.1 ml) was added to 0.1 ml of 4 *M* NaOH and 0.1 ml of internal standard (1.1 mg/ml phenacetin in water). The resultant solution was then extracted into 3 ml of 1:1 (v/v) dichloromethane–*n*-pentane. After the organic phase was separated, it was dried, then reconstituted in 200 μl of mobile phase (3:1 6.7 m*M* Na_2HPO_4, pH 7.2–acetonitrile). A 25-μl aliquot of this solution was injected onto the HPLC column (300 mm × 3.9 mm I.D., μBondapak C_{18}, 10 μm particle size, 30°C, Waters) and eluted isocratically at 1.7 ml/min. Detection was by UV absorbance at 254 nm. The method of peak-height ratios was used to calculate blood antipyrine concentration.

Calculations

Evaluation of fetal protein metabolism requires measurement of two of the three variables related as follows:

1. Synthesis = accretion (growth) + breakdown.

The following formulas can therefore be used to estimate the rate of fetal protein synthesis.

2. Synthesis (non-oxidative disposal) = leucine disposal − leucine decarboxylation

where leucine disposal is defined as:

3. Leucine disposal = (infusion rate − transumbilical loss)/specific activity.

In formula 3, transumbilical loss is:

4. Transumbilical loss = $UBF\{(dpm_{Leu\text{-}ua} - dpm_{Leu\text{-}uv}) + (dpm_{Kic\text{-}ua} - dpm_{Kic\text{-}uv})\}$

where UBF is umbilical blood flow and $dpm_{Leu\text{-}ua}$, $dpm_{Leu\text{-}uv}$, $dpm_{Kic\text{-}ua}$ and $dpm_{Kic\text{-}uv}$ represent the radioactivity as leucine and α-keto-isocaproate in the umbilical artery and vein. In formula 2, leucine decarboxylation is:

5. Leucine decarboxylation = $UBF(^{14}CO_{2ua} - {}^{14}CO_{2uv})$/specific activity

where $^{14}CO_{2ua}$ and $^{14}CO_{2uv}$ are the blood CO_2 radioactivity in the umbilical artery and vein respectively.

Fetal protein accretion can also be estimated because the only fates for leucine are either metabolism or accretion into protein. The difference between the uptake of leucine across the placenta and its rate of metabolism (oxidation) is, therefore, the rate of leucine accretion into protein.

6. Accretion = leucine uptake − leucine decarboxylation

where leucine uptake is:

7. Leucine uptake = $UBF\{([Leu]_{uv} - [Leu]_{ua}) + ([Kic]_{uv} - [Kic]_{ua})\}$

where $[Leu]_{uv}$, $[Leu]_{ua}$, $[Kic]_{uv}$ and $[Kic]_{ua}$ are leucine and α-ketoisocaproate concentrations in the umbilical vein and artery.

Statistics

Analysis of variance was used to dissociate intra-animal from inter-animal variability. Analysis of covariance was used to test whether there was any relationship between leucine or α-keto-isocaproate specific activities and the duration of the infusion of radioactive leucine. All results are presented as the mean ± S.D.

RESULTS

Successful measurement of fetal protein metabolism requires that leucine turnover (dispos-

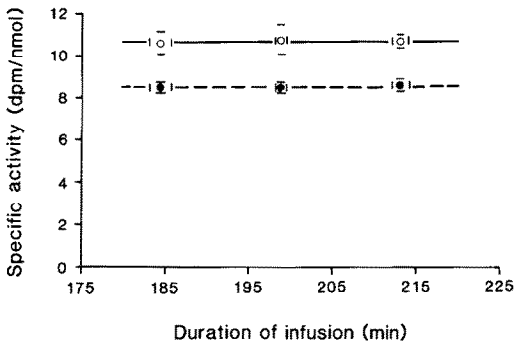

Fig. 3. Specific activity of plasma leucine (○) and α-ketoisocaproate (●) as a function of duration of infusion. Each data point is shown with S.D. after correction for inter-animal variability. The solid and dashed lines show for leucine (specific activity = 0.0033 × min + 10.05; $S.E._{slope}$ = 0.0092) and α-ketoisocaproate (specific activity = 0.0044 × min + 8.4; $S.E._{slope}$ = 0.0042), respectively, the regression equations derived by analysis of covariance. Neither leucine nor α-ketoisocaproate specific activity changed during the period from 180 to 210 min of infusion.

al), oxidation, and uptake be measured with sufficient accuracy to find the differences that may occur with changes in the fetal environment. In adults, leucine disposal can be calculated if the infusion of amino acids has reached steady state and one divides the known infusion rate by the specific activity of either leucine or its keto analogue, α-ketoisocaproic acid [5–7]. Fig. 3 shows that both leucine and α-ketoisocaproate specific activities reached constant specific activity by 180 min of infusion. After 180 min, not only was there no relationship between either leucine or α-ketoisocaproate specific activities and the duration of infusion, but 95% confidence limits on the slope included less than an 8% change in leucine specific activity and less than 5% change in α-ketoisocaproate specific activity over the period from 180 to 210 min of infusion.

Unlike adults, the fetus loses a significant proportion (20–40%) of the radioactive tracer across the umbilical circulation to the placenta and ewe [1,3,8,16,17]. We have elected to consider such transumbilical loss as separate from fetal leucine disposal [1]; others have included it as a separate component of fetal leucine disposal [3,8,16,17]. In either case this loss must be measured before the other components of fetal leucine disposal, oxidative and non-oxidative can be calculated. Table I shows the data needed for calculation of leucine disposal and its variability. Transumbilical tracer loss is calculated by the Fick principle as the umbilical blood flow times the umbilical venoarterial blood concentration difference for leucine radioactivity. There is a significant loss of

TABLE I

MEASUREMENT OF FETAL LEUCINE DISPOSAL

Variable	Unit	Mean	S.D.	C.V. (%)
Umbilical blood flow	ml/min/kg	212	24	11
Radioactivity infused	dpm/min/kg	132 409	12 220	9
Leucine radioactivity (artery)	dpm/ml	2046	204	10
Leucine radioactivity (umbilical vein)	dpm/ml	1895	197	10
Leucine radioactivity (VA difference)	dpm/ml	150	32	21
Leucine radioactivity lost	dpm/min/kg	30 540	6034	20
α-Ketoisocaproate radioactivity (artery)	dpm/ml	169	36	21
α-Ketoisocaproate radioactivity (umbilical vein)	dpm/ml	175	19	19
α-Ketoisocaproate radioactivity (VA difference)	dpm/ml	−4	9	231
α-Ketoisocaproate radioactivity lost	dpm/min/kg	−736	1952	265
Total activity lost	dpm/min/kg	30 354	8631	28
Leucine specific activity	dpm/nmol	10.68	0.76	7
Disposal (from leucine SA)	μmol/min/kg	9.98	0.86	9
α-Ketoisocaproate specific activity	dpm/nmol	8.52	0.66	8
Disposal (from α-ketoisocaproate SA)	μmol/min/kg	12.52	1.03	8

[^{14}C]leucine from the fetus across the umbilical circulation that represents 23.1 ± 9.1% of the fetal [^{14}C]leucine infusion. Because either the leucine or the α-ketoisocaproate specific activities may be used to calculate amino acid disposal rates (see Discussion), both are shown in the table.

Following conversion into α-ketoisocaproate, the next step in the degradation of leucine is decarboxylation. Because we infused [1-^{14}C]leucine, we could measure this rate of decarboxylation as the rate of CO_2 production by the fetus presuming that there is not a significant fixation of CO_2 in the fetal compartment, a fact that has been shown previously [18]. Table II shows that radioactivity as CO_2 is lost by the fetus. Leucine is not decarboxylated directly, but only after conversion into α-ketoisocaproate. Consequently, we have used plasma α-ketoisocaproate specific activity to convert this rate of CO_2 production into the rate of leucine decarboxylation. Such decarboxylation represents 15.9 ± 4.2% of the rate of leucine disposal (calculated using α-ketoisocaproate specific activity). This latter proportion represents then the proportion of leucine disposal that is metabolized (rather than synthesized into protein).

Leucine uptake across the umbilical circulation as calculated by the Fick principle is shown in Table III. Because there is a rapid equilibrium between leucine and its α-keto acid, we also measured the transumbilical uptake of α-ketoisocaproate so that we could use the sum of the uptakes of both leucine and its α-keto acid as a measure of fetal uptake of the leucine carbon skeleton. We were not able to show significant uptake of α-ketoisocaproate by the fetus. Expressed as a percentage, such uptake proved to represent only 8.5 ± 6.8% of leucine uptake. Consequently, although we have included both uptakes in our calculations, under the conditions of this experiment, leucine uptake could have been adequately quantitated by measurements of leucine uptake alone.

The variables that can be calculated from leucine disposal, decarboxylation, and uptake are shown in Table IV. Because leucine disposal rates may be calculated using either plasma leucine or plasma α-ketoisocaproate specific activities (see Discussion), there are two possible values for two of the three variables. Even though growth, and therefore protein accretion, is an important feature of fetal life, a significant proportion (78.1 ± 10.9%) of fetal leucine use for protein synthesis is not retained in accreted protein, but is instead recycled from protein breakdown (calculated from α-ketoisocaproate specific activities).

DISCUSSION

The purpose of this report is to outline methods for analysis of whole blood leucine and α-ketoisocaproate concentrations and radioactivity that are sufficiently accurate to be used to calculate the variables important to fetal protein metabolism. The methods outlined above were used on three separate occasions in four animals to allow us to quantify the within- and between-animal variability. Fetal protein synthesis, accretion, and breakdown were measured with C.V.

TABLE II

FETAL LEUCINE DECARBOXYLATION

Variable	Unit	Mean	S.D.	C.V. (%)
Umbilical blood flow	ml/min/kg	212	24	11
$^{14}CO_2$ (artery)	dpm/ml	256	17	7
$^{14}CO_2$ (umbilical vein)	dpm/ml	175	14	8
$^{14}CO_2$ (AV difference)	dpm/ml	80.8	7.5	9
$^{14}CO_2$ radioactivity lost	dpm/min/kg	16 273	1406	9
Decarboxylation (from α-ketoisocaproate SA)	μmol/min/kg	2.00	0.29	14

TABLE III

FETAL LEUCINE AND α-KETOISOCAPROATE UPTAKE

Variable	Unit	Mean	S.D.	C.V. (%)
Umbilical blood flow	ml/min/kg	212	24	11
Leucine (umbilical vein)	μmol/ml	239	20	8
Leucine (artery)	μmol/ml	219	17	8
Leucine (VA difference)	μmol/ml	20.0	3.8	19
Leucine uptake	μmol/min/kg	3.93	1.03	26
α-Ketoisocaproate (umbilical vein)	μmol/ml	24.0	5.6	23
α-Ketoisocaproate (artery)	μmol/ml	22.2	4.5	20
α-Ketoisocaproate (VA difference)	μmol/ml	1.8	1.5	81
α-Ketoisocaproate uptake	μmol/min/kg	0.35	0.29	83
Leucine + α-ketoisocaproate uptake	μmol/min/kg	4.28	1.18	28

values (within-animal) of 8, 49, and 20%, respectively.

The methods outlined in this paper are different from previously published data in several important respects. First, α-ketoisocaproate concentrations have been measured in whole blood. Previous reports have either detailed methods that are suitable for plasma [3,10,11,17] or that have required a larger blood volume than is practicable for fetal experiments [19]. Second, these methods of analyzing leucine and α-ketoisocaproate can all be done using HPLC with UV detection. Some other methods, though eminently suitable, require gas chromatography–mass spectrometry (GC–MS), a much more expensive and less available technology [3,17]. Third, we have presented a method of analyzing whole blood leucine and α-ketoisocaproate radioactivity, variables that are important in measuring the loss of radioactivity across the umbilical circulation, a critical variable for appropriate measurements of fetal protein metabolism.

Accurate determination of the three major components of fetal protein metabolism presumes an accurate determination of leucine disposal, oxidation, and uptake. Careful calibration of infusion pumps and measurement of infusate radioactivity introduces little variability to the measurement of infusion rate. The variability of measurement of plasma specific activities (7–8% in these experiments) introduces much of the measurement error. In fetal life, however, there is an additional important variable, the amount of radioactive leucine lost across the umbilical circulation. The loss of radioactive tracer to the placenta must be measured before quantities such as non-oxidative disposal of leucine (*i.e.* protein synthesis) can be appropriately calculated. We found a significant loss of tracer leucine across the umbilical circulation representing 23% of the

TABLE IV

FETAL PROTEIN METABOLISM

Variable	Unit	Mean	S.D.	C.V. (%)
Synthesis (leucine SA)	μmol/min/kg	8.48	0.73	9
Synthesis (α-ketoisocaproate SA)	μmol/min/kg	10.52	0.88	8
Accretion	μmol/min/kg	2.28	1.11	49
Breakdown (leucine SA)	μmol/min/kg	5.69	1.63	29
Breakdown (α-ketoisocaproate SA)	μmol/min/kg	8.24	1.63	20

radioactivity infused. Had we not measured this loss as significant, we would have overestimated the rate of fetal protein synthesis. The radioactivity lost across the umbilical circulation was calculated as the umbilical blood flow times the umbilical arteriovenous difference in leucine radioactivity. The accuracy of such measurements depends, in great part, on the ability to measure the arteriovenous difference — especially if that difference is small compared with the total intravascular concentration of the substance in question. In the present experiment the arteriovenous difference was 7.3 ± 1.9% of the arterial leucine radioactivity. Measurement of this relatively small difference contributed sufficient variability that the C.V. for radioactive leucine loss was 20%. Even so, the coefficient of variability for leucine disposal (*ca.* 10%) remained relatively low as the radioactivity lost contributed less than the measurement of specific activity to the variability of leucine disposal. It is difficult to compare the disposal rates reported here directly to other reports. During [1-^{14}C]leucine infusion, Loy *et al.* [3] measured leucine disposal rates of 8.97 ± 1.48 (C.V. = 17%) and 13.8 ± 2.3 (C.V. − 16%) μmol/kg per min at 130 days gestation using leucine and α-ketoisocaproate specific activities, respectively. From the same laboratory, Kennaugh *et al.* [8] reported disposal rates of 16.1 ± 1.5 μmol/kg per min (C.V. = 31%) at *ca.* 80 days gestation. To compare either of these reports with the present, their disposal rates would need to be adjusted for the loss of radioactive leucine across the placenta (*ca.* 40% of that infused). Our disposal rates are therefore higher than the first report but comparable with the second. They are also similar to those reported at 120–125 days gestation by Liechty *et al.* [20] of 10.1 ± 3.0 μmol/kg per min (C.V. = 30%; weight estimated from autopsy data) who used GC–MS. However, these investigators were not able to measure the loss of leucine radioactivity across the placenta, and, as a consequence, their estimate may be 20–40% higher than the actual turnover rate.

To calculate fetal accretion of leucine, the rate of leucine and α-ketoisocaproate uptake by the fetus across the umbilical circulation must also be measured accurately. Leucine uptake was measured in whole blood samples as 3.9 μmol/kg per min with a C.V. of 26%. This value is comparable with previous reports (3.7–5.1 μmol/kg per min; C.V. 13–109%) [20–22]. Most of the variability in this measurement is caused by the relatively small venoarterial difference that must be measured compared with the arterial concentration of this substrate (*i.e.* the difference is 9.0 ± 1.5% of the arterial concentration). Ketoisocaproate uptake (0.35 ± 0.29 μmol/kg per min) proved even more difficult to measure accurately (C.V. = 83%). Others have had similar difficulty (0.20 μmol/kg per min; C.V. = 300%) [20]. Again the difficulty was not in measuring umbilical blood flow (C.V. = 11%), but rather in measuring a whole blood venoarterial difference that was 7.4 ± 4.9% of the arterial concentration. However, in these studies, α-ketoisocaproate uptake proved to be *ca.* 9% of leucine uptake. The inaccuracy of the total uptake of leucine and its α-keto analogue was, therefore, more related to the smaller inaccuracy in measurement of leucine uptake. Indeed, one could eliminate α-ketoisocaproate measurements entirely without materially affecting the remaining calculations.

The fetal blood volume is *ca.* 360 ml at the gestational age of the animals used in this study. Disturbances of fetal homeostasis have been described when as little as 3% of the fetal blood volume is removed. We are therefore constrained to *ca.* 11 ml of total blood sampling without replacement. The blood sampled to make all the measurements needed to describe protein metabolism is less than 10 ml, thereby meeting this constraint. We were able to minimize blood sampling volume because we used one 0.5-ml blood sample to measure whole blood concentrations and radioactivity of both leucine and α-ketoisocaproate. In spite of these measures to conserve blood volume, however, the volume of blood needed for each measurement of protein metabolism is sufficient that serial measurements of protein metabolism will require replacement of the removed fetal red cell mass between measurements to prevent a fall in fetal hematocrit.

The appropriate specific activity that should be sampled to estimate the various elements of leucine disposal remains controversial [1,2]. To estimate leucine use for protein synthesis, for example the specific activity of the immediate precursor to protein synthesis, leucyl-tRNA, should be measured. Because measurement of this particular pool has proven difficult [2], other, more accessible, leucine pools have been sampled. The two most obvious pools for such sampling are the intracellular and extracellular leucine pools. Unfortunately, which of these two pools best represents the specific activity of leucyl-tRNA remains unclear. Although intracellular amino acids would seem to be the logical precursors for aminoacyl-tRNA, in some organs extracellular amino acid specific activity seems to be more closely related to that of aminoacyl-tRNA [2,23,24]. Extracellular amino acid specific activity is generally sampled as plasma leucine. Intracellular pools may be sampled indirectly by measuring the specific activity of plasma α-ketoisocaproate, the α-keto analogue that equilibrates rapidly with the intracellular leucine pool [2,7]. Because of the uncertainty as to which of these two specific activities is the more appropriate for estimating whole body protein synthesis (indeed the specific activity of the protein synthetic pool may lie somewhere between the two), we have presented calculations of protein metabolism using both.

Experiments that use each animal as its own control eliminate the contribution of inter-animal variability to the measurements, thereby markedly decreasing the number of animals needed to find effects due to changes in independent variables. For example, the non-oxidative use of leucine (fetal protein synthesis) estimated from α-ketoisocaproate specific activity has a C.V. of 8% when estimated from the variation within animals. If the intra-animal variability is included, the C.V. is 13%. Detection of a 10% difference in the rate of fetal protein synthesis would require eight pairs of measurements (eight animals) using each animal as its own control and seventeen pairs of measurements (thirty-four animals) if only a single measurement is possible in each animal. Obviously, the ability to make repetitive measurements in the same animal would allow experiments that would otherwise be inordinately expensive and wasteful of animals.

In summary, we have developed methods for measuring whole blood concentrations of leucine and α-ketoisocaproate as well as methods for quantitating leucine and α-ketoisocaproate radioactivity in whole blood samples. These methods are sufficiently sensitive that requirements for blood volume should not alter fetal homeostasis. As a consequence, measurements of fetal protein metabolism can be made with sufficient accuracy to detect differences as small as 10% in variables such as fetal protein synthesis provided appropriate experimental designs are used.

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