NITROGEN METABOLISM IN PRETERM INFANTS

Cover illustration: Schematic representation of amino acids. Nitrogen and first carbon atom are labelled.

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NITROGEN METABOLISM IN PRETERM INFANTS

(STIKSTOF METABOLISME BIJ PREMATURE PASGEBORENEN)

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
Prof. Dr. P.W.C. AKKERMANS, M. Lit.
EN VOLGENS HET BESLUIT VAN HET COLLEGE VAN DEKANEN.

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GEBOREN TE LAREN (N.H.)

PROMOTIE-COMMISSIE

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OVERIGE LEDEN:	Prof.Dr. H.J. Degenhart
	Prof.Dr. A. Okken
	Prof.Dr. J.H.P. Wilson

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Dit boek is voor mijn vader, mijn moeder en voor Marjan

De eerste bekende intraveneuze toediening van voeding vond plaats in 1656, bijna 350 jaar geleden. Sir Christopher Wren injecteerde oplossingen van "voedende stoffen" en medicijnen in honden. Daartoe gebruikte hij een holle ganzepen die verbonden was met een varkensblaas. De oplossingen die hij toediende bestond uit bier, opium en wijn. In 1667 gaf Denis bloed van een lam aan een mens. Dit kan worden beschouwd als de eerste toediening van eiwit cq amino zuren aan de mens. Het leverde toentertijd niet het gewenste resultaat op. Vele patienten overleefden de behandeling niet.

CONTENTS

VOORWOORD

Het boek dat nu voor u ligt, staat bol van onderzoek. Vroeger kon wellicht een groot deel van het promotie-onderzoek in totale afzondering op een duister kamertje worden bedacht, uitgevoerd en opgeschreven, maar tegenwoordig is dat duidelijk niet meer het geval. Onderzoek vindt plaats in grote ruime laboratoria en afdelingen, wordt bedacht in grote, soms overvolle, kamers met veel computers en het belangrijkste: met een groot aantal mensen. Onderzoek, en zeker klinisch onderzoek, is tegenwoordig "team-work". Daarom wil ik de tientallen mensen bedanken die de afgelopen jaren zich hebben ingezet voor dit onderzoek.

De verpleging van de afdeling Pasgeborenen, niet in de laatste plaats voor het alsmaar weer plakken van zakjes; daarnaast de medische staf van Pasgeborenen, de medewerkers van het wetenschappelijk laboratorium, de medewerkers van het klinisch chemisch lab en van het lab Interne II op Dijkzigt, medewerkers van de apotheek, allen ben ik zeer erkentelijk. Daarnaast wil ik nog een aantal mensen persoonlijk noemen.

Prof. Dr. Pieter J.J. Sauer, promotor, is een promotor zoals iedere AIO/OIO zich mag wensen. Letterlijk elke dag klaar staan om te helpen, om problemen aan te horen en de richting aan te geven waar de oplossing gezocht moet worden. Op congressen me overal mee naar toe nemend, tot en met etentjes met de "internationale kopstukken". Maar ook door me mijn gang te laten gaan en me vrij te laten waar het kon. Ik heb een prachtige tijd gehad, waarvoor jij voornamelijk verantwoordelijk bent geweest.

Prof. Dr. Herman J. Degenhart, zie ik als een professor in de klassieke zin van het woord. Iemand die van de meest uiteenlopende zaken verstand heeft en daar graag over discussieert. Altijd de deur open om mijn vragen te beantwoorden en om bijvoorbeeld onze hersenen te pijnigen omtrent de essentialiteit van glycine.
Naast een warme belangstelling op het persoonlijke vlak, altijd bereid om manuscripten te verbeteren, statistische hulp te bieden en, niet onbelangrijk, dit proefschrift zeer grondig te lezen.

Prof. Dr. A. Okken en Prof. Dr. J.H.P. Wilson dank ik voor hun bereidheid het manuscript kritisch te beoordelen en tevens het zo snel te doen zodat ik toch nog kan promoveren voordat ik met de opleiding Kindergeneeskunde begin.

Dr. Eric J. Sulkers, collega van het eerste uur. Veel voorbereidend werk was al door jou gedaan, zodat ik eigenlijk in een gespreid bedje terecht kwam. We hebben erg plezierig samengewerkt en veel in de kroeg gezeten om in een andere omgeving over onderzoek en wat dies meer zij te praten. Gezien de vele uren die we in die tijd aan onderzoek besteden, heb ik er alle hoop op dat we dit ook tijdens de opleiding vol moeten kunnen houden.

Dr. Virgilio P. Carnielli, the fat-man. Although neglecting the most important issue in life (protein), you have shown me how to set up metabolic studies in which nothing has been forgotten. I have really enjoyed working with you and hope that you and Paola will stay for a long time in a country where you really appreciate the moments the sun is shining.

Research verpleegkundige Anneke A. Boerlage laat duidelijk zien dat met een goede organisatie en nauwgezetheid, een enorme hoeveelheid werk kan worden verricht. Jij hebt tevens het aloude gezegde "voor wat, hoort wat" in ere hersteld. Sinds jouw komst worden de kinderen in het onderzoek verwend met beertjes, andere beestjes, handige flessen en worden ze, nog meer dan vroeger, geknuffeld.

Hoofdanaliste Ingrid H.T. Luijendijk is een onmisbare steun geweest in het lab. Jij hebt mij de ins en outs van vele bepalingen geleerd, waaronder natuurlijk de bepaling met de Conway-Lips schaaltjes. Altijd op de hoogte waar alles ligt en altijd met een goed humeur.

Massa-spectrometrie expert J.L. Darcos Wattimena heeft mij het werken op apparaten die even duur zijn als villa's in Wassenaar geleerd. Dat ging niet altijd zonder problemen, maar dankzij jouw hulp zijn alle bepalingen uiteindelijk verricht.

Prof. Dr. Harrie N. Lafeber, lid van de promotie-commissie en officiële begeleider in het eerste jaar, heeft in het begin geholpen met de uitvoering van de studies in de research-couveuse. Een grote "multi-center study" is nu van start gegaan zodat we ongetwijfeld nog contact zullen houden.

Paul A.J.M. Adan, analist, je hebt me erg geholpen met alle CO_2 bepalingen, een soort lopende band werk waarbij ik de monsters opwerkte bij de glasblazers en jij de verrijking bepaalde. Doordat jij altijd zeer vroeg in de ochtend startte, hebben we heel wat effectieve dagen van de IRMS gebruik gemaakt.

Robin Jankie, analist, heeft alle aminozuur bepalingen gedaan, tussen de gewone bepalingen in en dat altijd zeer snel. Samen met de interpretatie van Jan G.M. Huijmans betekende het dat de manuscripten snel de deur uitkonden.

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Zonder geld geen zaken. Riet Visser en Jacqueline de Vogel hebben alle geldstromen in uitstekende banen geleid en waren niet bijzonder boos wanneer ik weer eens een pakje had verzonden in plaats van dit via hun te doen (alsof ik het nooit leerde).

Joop van Dijk, grafisch ontwerper, heeft de voorkant van het proefschrift gemaakt en zelfs vervaagde plaatjes uit 1948 met de scanner weer nieuw aanzien gegeven.

Rotterdam heeft een medische faculteit die het onderzoek een zeer warm hart toedraagt. Er zijn dan ook vele studenten die elk voor een deel hebben bijgedragen aan het onderzoek: Casper Leunisse, Jeroen Adan, Monique Achterberg, Thomas Colen, Michelle Timmerman, Michel Kenters, Wilma Huisman.

Met plezier werken houdt niet alleen in dat je werk leuk is, ook de mensen met wie je omgaat moeten plezierig zijn. Arjen, Arne, Bernadette, Corinne, Henriette, Marja, Niels, René, Wouter, Yolanda, ik ben altijd met veel plezier naar mijn werk gegaan.

Te vroeg geboren kinderen, om hen is het allemaal te doen. Alle ouders die toestemming voor het onderzoek gaven wil ik bedanken dat zij, door hun kinderen te laten participeren, meehielpen om de behandeling van deze kwetsbare groep kinderen te verbeteren. En dat terwijl er vaak geen direct voordeel voor hun kind bestond, erger nog het betekende meestal extra bloedafnames, extra handelingen, terwijl hun kind al zo ziek was. Hen ben ik zeer erkentelijk.

Mijn ouders dank ik voor de uitstekende opvoeding en alle kansen die zij mij gaven.

Marjan dank ik het meest, voor haar onvoorwaardelijke steun.

CHAPTER 1

INTRODUCTION

INFANTS

Live born infants delivered before 37 weeks from the first day of the last menstrual period are termed *premature* by the World Health Organization [1]. Historically, prematurity was defined by a birth weight of 2500 grams, or less. Today infants who weigh less than 2500 grams at birth are termed "low birth weight (LBW) infants". LBW infants can be premature (<37 wk of gestation), intra-uterine growth retarded for their gestational age, or both. Both prematurity and intra-uterine growth retardation are associated with increased neonatal morbidity and mortality. During 1991, 11.2% of the live births in the Netherlands weighed less than 2500 grams, whereas 11.9% of the live births were born prematurely [2]. Very low birth weight (VLBW) infants weigh less than 1500 grams and are predominantly premature. In the Netherlands in 1991, 2.0% of the live births had a very low birth weight.

VLBW infants account for approximately 66% of the deaths in the first week of life; their survival is directly related to their birth weight, with the survival rate being approximately 20% for those between 500-600 grams and 85-90% for those between 1250-1500 grams [1]. The greater knowledge of neonatal medicine, accompanied by improved technical possibilities in treating neonatal diseases has resulted in a dramatic rise in survival of VLBW infants. With the decreased mortality rate, an increase in the morbidity rate is seen. The vast majority of VLBW infants is unable to survive without intensive care because of the immaturity of anatomic structures or biochemical and physiologic functions. They are cared for at or near a neutral thermal environment by keeping them in incubators to conserve body heat. Most VLBW infants need additional oxygen due to pulmonary problems.

GROWTH

Most VLBW infants face many diseases during their neonatal period and thereafter, which affects their growth. Between 26 and 36 weeks of gestation the weight gain of the fetus is higher than at any other time thereafter in life. Average weight gain is 15 - 17 gram per kilogram per day, with a threefold increase of weight within this period. The central nervous system continues to grow until one year after birth. Adequate nutrition in the neonatal period is therefore very important for growth and development. Recent studies show a clear effect of the type of feeding in early life on psychomotor and neurological development of VLBW infants at 7 years of age [3,4].

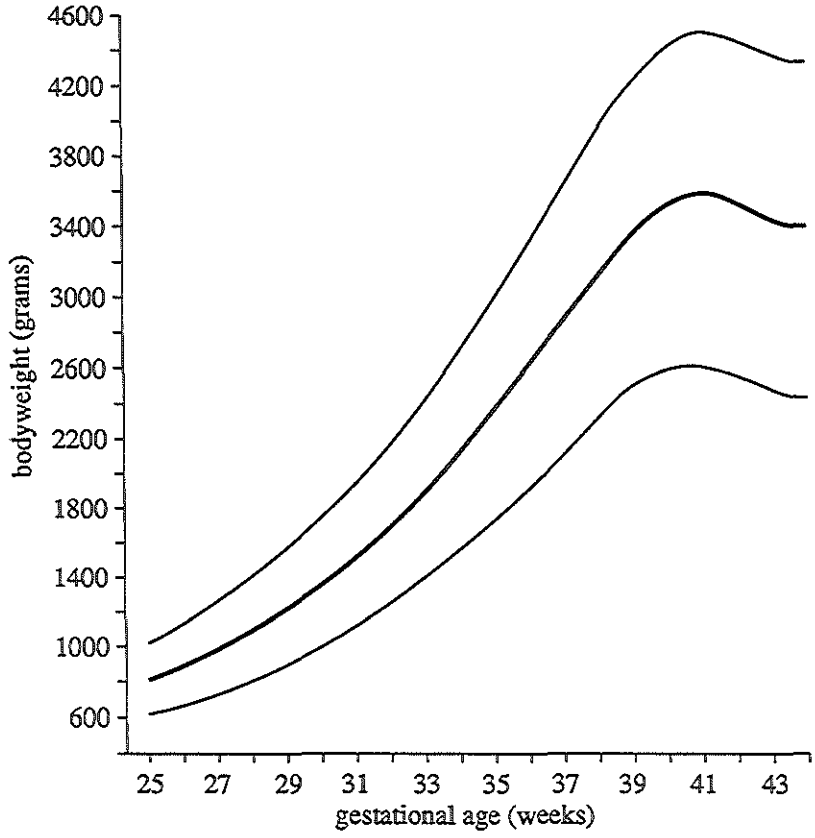
The immaturity and clinical problems compromise and complicate the provision of adequate nutrition. Breast or bottle feeding for VLBW infants is usually not possible. It requires the coordination of swallowing, epiglottal and uvular closure of the larynx and nasal passages, and normal oesophageal motility. This synchronized process is usually absent before 34 weeks of gestation. Furthermore there are indications that delaying early introduction of oral feeding reduces the incidence of necrotizing enterocolitis, a life-threatening disease [5]. Parenteral nutrition seems therefore the suitable way to provide nutrients to the preterm infant during the direct postnatal phase, when enteral feeding is thought not to be advisable.

Why should we provide nutrients to the infants in the direct postnatal phase?

During fasting the neonate derives energy from body stores for continued functioning. The first store used is liver glycogen, which is limited in amount and rapidly depleted. Thereafter, fat stores become the most important source for providing calories, but protein stores are also used to provide amino acids for gluconeogenesis and to supply additional fuel by direct oxidation amino acids. 1-5% of the weight of VLBW infants consists of fat and about 8-10% consists of protein. Thus, the VLBW infant has limited energy reserves to survive. Without nutrient supply the survival time of VLBW infants has been estimated to be four or five days [6]. Daily provision of exclusively glucose will prolong survival time for about two weeks [7]. The depletion of body protein limits the survival under circumstances of inadequate fuel supply. So without appropriate nutritional support the survival time is very limited.

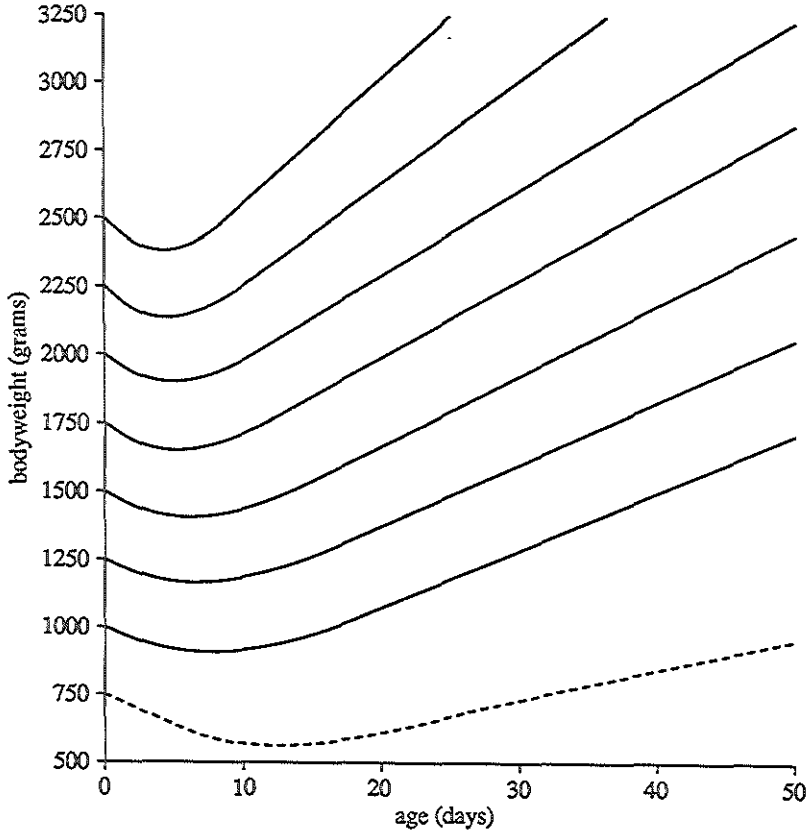
As discussed above, fetal growth and development accelerates in the third trimester of pregnancy. This *in utero* effect is potentially lost to the infant born significantly preterm. Adequate nutrition should be provided to obtain similar growth rates as in utero, without evident metabolic disbalances. This goal has been postulated by the American Academy of Pediatrics in 1985 [8]. Although the validity of this goal is still debated, no other acceptable alternative has been proposed. **Figure 1.1** shows the weight gain of the fetus in utero. Term infants usually face a short period of weight loss following birth. Stable preterm infants demonstrate a longer period of weight loss (**Fig 1.2**). The curves shown in this figure are based upon data from 1948, but they are still valid although knowledge and treatment of perinatal diseases has improved dramatically [10]. Growth retarded infants however seem to gain weight significantly more rapidly compared to appropriate for

Figure 1.1 Weight of the fetus (mean±2SD) in utero during pregnancy (From Usher and McClean [9], with permission)



gestational age preterm infants, but this is not true for infants with a birth weight of less than 750 g [11]. As can be expected, the degree of illness is an important parameter of the time needed to regain birth weight and the rate of weight gain thereafter. Infants with severe hyaline membrane disease at birth might develop a chronic lung disease called bronchopulmonary dysplasia as the result of mechanical ventilation and oxygen toxicity. Those infants are particularly susceptible to growth failure (**Fig 1.3**). Furthermore, other complications such as infections, metabolic diseases and endocrine disorders lead to a reduced weight gain.

Figure 1.2 Grid for recording weights of premature infants. The average weight increments are indicated on the basis of weight at birth. (From Dancis *et al.* [10], with permission)



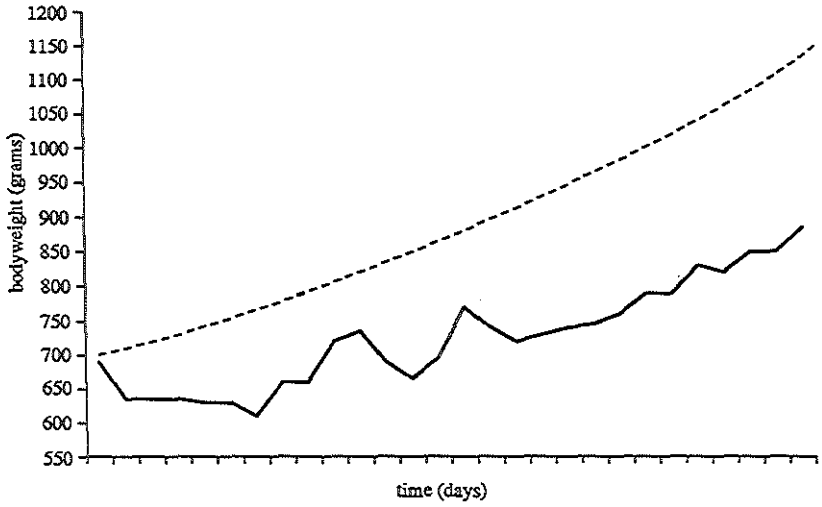
NITROGEN METABOLISM

As depletion of protein is one of the limiting factors of survival, the accretion of body protein is the most important factor for growth if there is an excess of nutrients [12]. It is not surprising, therefore, that there is a general interest to learn more about the nature of protein metabolism, and in quantifying the magnitude of protein kinetics in growing infants.

Performing a nitrogen balance is the conventional method of assessing changes in body protein status. By simply measuring the amount of nitrogen entering and leaving the body, the overall retention can be calculated. Using this method, estimates of protein requirement are made and the nitrogen mass balance technique has supplied us with invaluable data. Individual amino acid requirements are determined in this way as well [*e.g.* 13,14]. In these studies, neonates were fed mixtures of free amino acids based on the pattern of mother's milk protein. Following withdrawal of one specific amino acid, this amino acid was introduced again. The amount needed to reach a similar nitrogen retention as with the complete mixture was considered to be the minimal requirement of that amino acid [15]. Besides limitations as unmeasurable losses, no information is given on the dynamic feature of nitrogen metabolism. The discovery of the dynamic nature of protein in the body can be attributed to Schoenheimer and associates in the 1930s [16,17]. By using stable isotope labelled tracer, they discovered that proteins in the body are continually being broken down and resynthesized. With this technique it became possible to study the two components affecting nitrogen balance; protein synthesis and protein breakdown.

Although stable isotopes have been applied for the last 55 years to study protein turnover, technical difficulties prevented its wide-spread use during the first three decades. During the last two decades, the use of stable isotopes to study metabolic processes has increased tremendously. This is attributed to a few fundamental advances in the analysis of stable isotope enrichment by mass spectrometry (quadrapole gas chromatograph-mass spectrometer and selective ion monitoring) and to an increased awareness of the health hazards of radioactivity. The intensified research has given us much insight into protein synthesis and breakdown and on the factors influencing these processes. Most of the studies have been performed on animals or human adults, data on neonates is still scarce.

Figure 1.3 Example of a weight curve of a small for gestational age infant with bronchopulmonary dysplasia. The dotted line represents the intra-uterine weight gain.



NITROGEN TURNOVER; TECHNIQUES

In neonates it is ethically hardly defendable to obtain a sample of structural protein (e.g. muscle) for direct isotope analysis as is often done in adults or animals. Methods for studying protein metabolism in neonates have to be performed in the least invasive way possible. Radioactive tracers are not used in infants because of the potential detrimental health effects. The ionizing radiation may cause injury to the molecular structure of the cell, leading to chromosome aberrations or cell death. The most obvious advantage of stable isotopes is that they are non-radioactive and present little or no risk to human subjects [18]. Toxicity of stable isotopes have only been found in animals receiving more than hundredfold higher dosages than given to humans. No toxity have been found in humans in 50 years of stable isotope usage. Side effects of high dosages of deuterium (vertigo) in humans have been reported but this is a transitory phenomenon, with no demonstrated evidence of permanent effects [18].

Additionally, it is possible to study nitrogen metabolism itself with ^{15}N, whereas there is no radio-active tracer of nitrogen. With the present available

techniques, the enrichment is measurable in small samples of blood. Another advantage of stable isotope tracers is that it is possible to use simultaneously different tracers or repeated tracers in the same subject. Assumptions when using stable isotope dilution techniques include the following:

1. The labelled molecule will not be discriminated from the unlabelled molecule.
2. The labelled molecule will trace the movement of the unlabelled molecules.
3. The administration of the labelled molecules will not affect the kinetics of the unlabelled molecules.

Small isotopic effects have been reported in micro-organisms [19,20], but the errors involved in the determination of nitrogen kinetics *in vivo* are much larger, so that small isotopic effects are not a factor of importance.

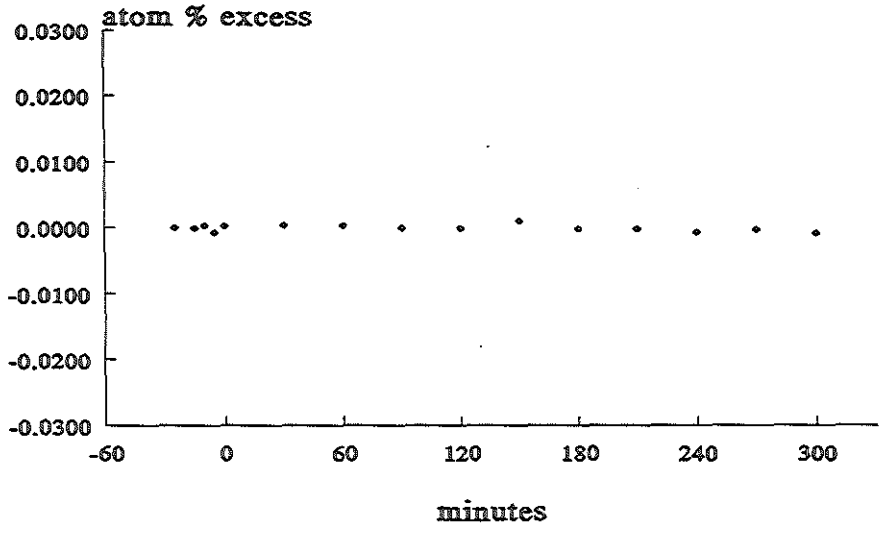
Natural enrichment

The natural abundance and isotopic enrichment attained in neonates for the two stable isotopes (^{13}C and ^{15}N) during the studies in this thesis are shown in **table 1.1.** More than 1% of all carbon atoms consist of the stable isotope ^{13}C, which means that about 2 grams of carbon atoms per kilogram body weight are labelled. Following stable isotope studies, this number will increase towards 2.015 g ^{13}C per kilogram body weight. The natural abundance in enrichment of stable isotopes is normally accounted for by taking baseline samples prior to the start of the isotope infusion and then subtracting the enrichment of the baseline sample from all the samples obtained during and/or after the isotope infusion.

Table 1.1. Natural enrichment and isotopic enrichment during stable isotope tracer experiments.

isotope	Natural enrichment		Tissue enrichment
	%	mg/kg body wt.	mg/kg body wt.
^{13}C	1.111	2000	2015
^{15}N	0.360	110	120

Figure 1.4 Isotope enrichment of expired CO_2 without tracer infusion on the first day of life.



This is satisfactory for experiments in which no change in metabolic status occurs during the study, but it may lead to serious errors if, for instance, the treatment intervenes with the metabolic situation. For instance, a shift from the utilization of endogenous fat to glucose will alter the isotope enrichment of expired CO_2, because of the difference in the naturally-occurring enrichment of carbohydrate and fat [21,22]. By keeping the quality and quantity of the nutrient intake to preterm infants constant as is done for most of the studies presented in this thesis, it can be assumed that the variation in background is negligible. For studies on the first day of life, it is only possible to influence the nutritional intake from birth onwards, while major metabolic adaptations take place in the direct postnatal period. To exclude changes in background enrichment of expired CO_2, we measured the enrichment of expiratory air of infants on the first day of life, while the infants did not receive stable isotope tracers. An example of such a study in a mechanically ventilated infant receiving exclusively glucose is shown in **Figure 1.4.** No significant changes were noticed over a period in which usually a stable isotope study is performed.

Infusion techniques

To determine substrate kinetics with stable isotopes, tracers can be delivered as a single bolus or as an infusion at a constant rate.

The major advantage of the single bolus technique is that substrate kinetics can be calculated through different compartments in the body. The major disadvantage of using bolus injections is that multiple samples have to be taken from different compartments, which make the studies ethically difficult to perform in preterm neonates. Another limitation when using bolus injections of stable isotope tracers lies, in contrast to radio-active labelled tracers, in the sensitivity and precision in the determinations of enrichment. The later points are especially important after the administration of a tracer bolus, but the enrichment is low at that time. To be able to confidently measure later time points after the bolus administration, large amounts of tracer have to be given. However, the amount of tracer needed in such studies could then be exceeding a tracer dose and influence the kinetics of the tracee [23].

With the constant infusion technique, no information is obtained on different compartments and fluxes through those compartments. On the other hand, once an apparent equilibrium is achieved following constant infusion, the nature and complexity of the volume of distribution of the substrate is not an issue. The technique also limits the amount of samples to be taken to a minimum, which is very attractive for studies in preterm neonates.

For the reasons listed above, all studies presented in this thesis are performed using the constant infusion technique.

Reaching plateau

When a labelled substrate enters a metabolic pool by continuous infusion, eventually a plateau in enrichment will be achieved (**Fig 1.5, 1.6**). The isotope enrichment of the infused pool will gradually rise until the same enrichment is reached in all sites of deposition. Aub *et al.* calculated that it will take about 40 days to get all amino acid pools in equilibrium in the rat [24]. Tracer studies usually only last a few hours for practical reasons, indicating that no real equilibrium is obtained, but an apparent or pseudo-plateau. However the deviation of the plateau from the asymptotic value can be regarded as negligible [25].

Figure 1.5 Schematic representation of stable isotope dilution technique. Adapted from RR Wolfe [23], with permission. Schematic representation of a single pool with Ra (rate of appearance) coming from constant infusion of tracee. In a steady state, Ra = Rd (rate of disappearance). Open circles correspond with the unlabelled molecules, closed circles with the labelled molecules. A, before tracer infusion; B, immediately after start of tracer infusion; C, some time later; D, at isotopic equilibrium.

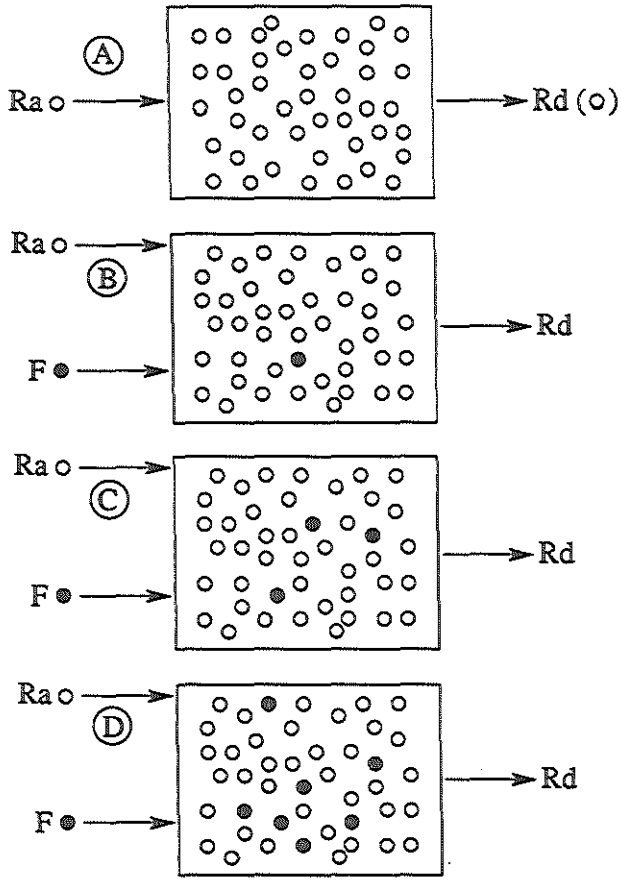
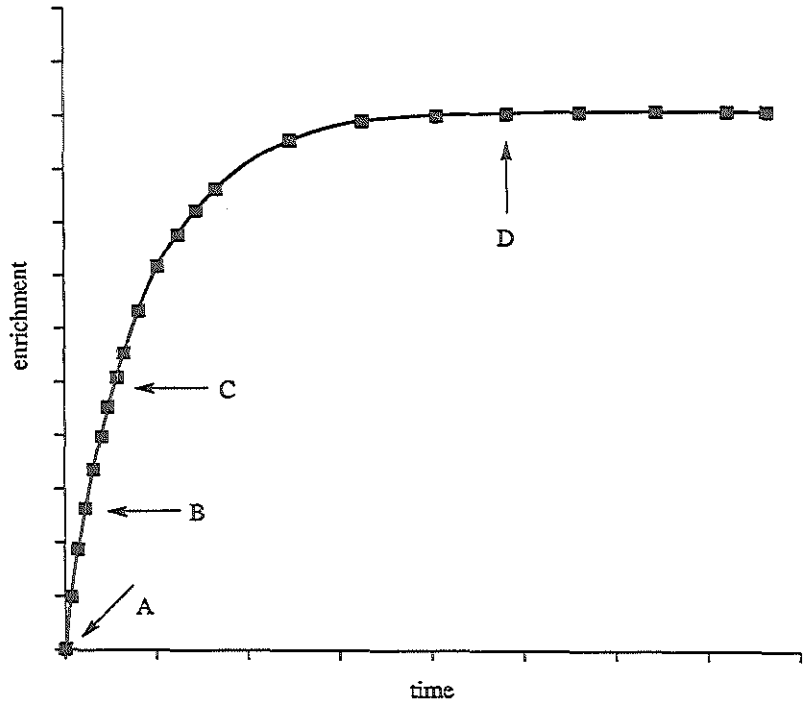


Figure 1.6 Course of the stable isotope enrichment during continuous infusion. Adapted from RR Wolfe [23], with permission. Letters refer to parts A, B, C, and D in figure 1.5.

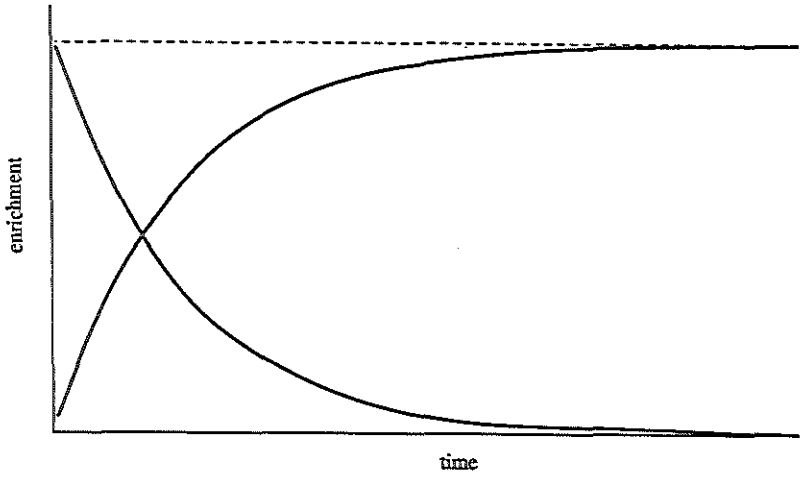


If the pool size is relatively large compared to the turnover rate, it may take several hours, even days to reach an apparent equilibrium during a constant infusion. This might be undesirable because of the practical consequences of performing a study and keeping the subject in a constant metabolic state. Another undesirable effect of a long duration of stable isotope tracer studies is recycling of the label.

Recycling of label

Tracer incorporated in larger substances (*e.g.* amino acids into proteins) might be released after some time and subsequently give rise to a higher enrichment.

Figure 1.7 Course of substrate enrichment following primed continuous infusion. The dotted line represents the resulting enrichment of the decay of enrichment of the priming dose and the rise in enrichment from the continuous infusion. Adapted from RR Wolfe [23], with permission.

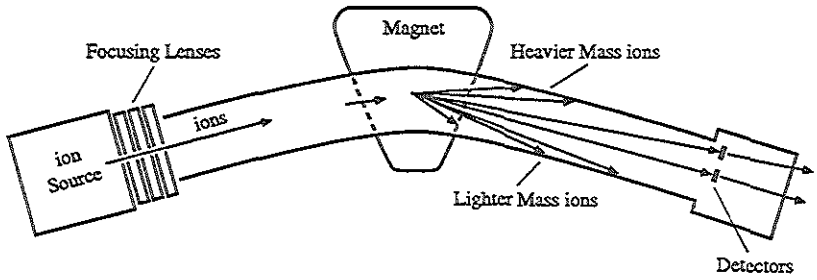


For leucine this phenomenon has been estimated to overestimate enrichment by 30% in 24 hours [26]. The duration of an experiment should therefore be kept to a minimum to reduce the recycling of the label. The time it requires to reach an apparent isotopic equilibrium can be shortened by injecting a priming dose of tracer just before beginning the infusion.

Priming the pool

The goal of a priming dose followed by a constant infusion is to label the pool instantaneously at such an enrichment that would have been reached after prolonged continuous infusion without a priming dose (**Figure 1.7**). Ideally an isotopic equilibrium could be achieved within minutes, and this has been shown for bicarbonate [27]. The use of a priming dose does not influence the final plateau enrichment, but only shortens the time to reach a plateau [28,29].

Figure 1.8 Schematic figure of principle mechanism of mass spectrometry. Adapted from RR Wolfe [23], with permission.



Determination of istopic enrichment

The isotopic enrichment of a compound is measured by mass spectrometry. There are several types of mass spectrometers. Depending on the compound either an isotope ratio mass spectrometry (IRMS) or a gas chromatograph mass spectrometry (GC-MS) can be used. Recently a combination of both systems has become available (GC-IRMS).

A commonly used IRMS system measures isotopic enrichments of elements in gaseous form. Therefore only gas molecules as CO_2, N_2, H_2 and SO_2 can be measured. Within a high vacuum, gas molecules stream from the inlet into the source of the mass spectrometer. The molecules are bombarded by electrons that are emitted from a filament wire. The gas molecules become positively charged molecular ions by the bombardment, because they lose an outer electron. The ions are led through an electromagnetic field at high velocity. Due to their mass and charge, they are deflected when passing through the magnetic field. **Figure 1.8** shows an illustration of the principle. Due to the relatively large difference in mass of each molecular ion, the isotopes can simultaneously be collected on spatially separate detector plates. As the ion beam hits a collector, an electron current is dyscharged. The ratio of for instance ion currents generated from the impact of ions of $^{12}CO_2$ (mass 44) and $^{13}CO_2$ (mass 45) can be measured, which is directly related to the isotopic ratio.

The most frequently used IRMS systems are only able to measure the enrichment of a limited number of pure gases. With a GC-MS, an almost unlimited variety of samples can be analyzed, but the precision is decreased (Table 1.2). The principle of a GC-MS is that the GC provides a simple and reproducible method for separation of volatile compounds. After the separation of the compounds, at the end of the analytical column, the compound of interest is brought into the ion source of the mass spectrometer. Here the molecule is ionized either by electron impact with a high fragmentation rate of the molecules or by chemical ionization, which leads to more intact ionized molecules. Again the molecules or fragments are selected on mass, either by a quadrupole mass filter, or by a double focussing magnetic sector mass.

PROTEIN TURNOVER; CALCULATIONS

The turnover or flux describes the process of replacement or renewal of a given substance [30]. If the patient is in steady state, the amount of substrate entering the pool equals the amount leaving the pool. To calculate turnover rates from continuous tracer infusion studies, samples are taken from the pool in which isotopic equilibrium is reached. The turnover of a substrate is determined by the dilution of the tracer. There are two approaches to measure whole-body protein turnover. The classical approach uses a carrier amino acid (usually [^{15}N]glycine) to bring labelled nitrogen in the nitrogen pool in the body. Nitrogen metabolism itself is being studied. The second approach measures the turnover of one amino acid and assumes that the turnover of this specific amino acid is representative of whole-body protein turnover. Both methods are compared and extensively discussed in chapter 3, but the basic concepts are given below.

Classical approach

Protein turnover can be determined by measuring the dilution of ^{15}N, the only tracer available for nitrogen. The dilution of ^{15}N can be measured in urinary ammonia or urea, which should be in equilibrium with the plasma compounds. It is therefore a non-invasive method, advantageous for studies in preterm neonates. The dilution of the tracer is inversely related to the turnover rate.

Table 1.2 Comparison GCMS and IRMS.

	GCMS	IRMS
Measurable tracer enrichment (Atom % Excess)	0.2% - 100%	0% - 0.5%
Amount of tracer needed	picograms	micrograms
Precision	± 0.2%	± 0.003%

It is assumed that the body has a single nitrogen pool with a constant size, implying that nitrogen entering the pool equals nitrogen leaving the pool. Nitrogen can enter the pool through the diet or breakdown from proteins. Nitrogen might leave the pool *via* excretion and *via* incorporation of nitrogen into proteins. In equation:

$$Q(flux) = Intake + Breakdown = Excretion + Synthesis$$

This mass balance equation can be solved by measuring nitrogen intake and excretion if the nitrogen flux is known.

Representative amino acid approach

The turnover rate of an individual amino acid can be extrapolated to whole-body protein turnover rate, considering that body protein has a fixed amount of each amino acid. Although ^{15}N can be used as labelling, also other stable isotopes like ^{13}C and deuterium are possible. The turnover of the amino acid is determined by the dilution of the tracer in constant infusion studies according to the following equation:

$$Turnover = \left(\frac{IE_{infusion}}{IE_{pool}} - 1\right) \times I$$

where $IE_{infusion}$ is the isotopic enrichment of the infused tracer (usually >98%) , IE_{pool} is the isotopic enrichment of the tracer in the measured pool and I is the infusion rate of the tracer.

By extrapolating the amino acid turnover rate to whole-body protein turnover rate and using the first equation described on the previous page, whole-body protein synthesis and breakdown rates can be obtained.

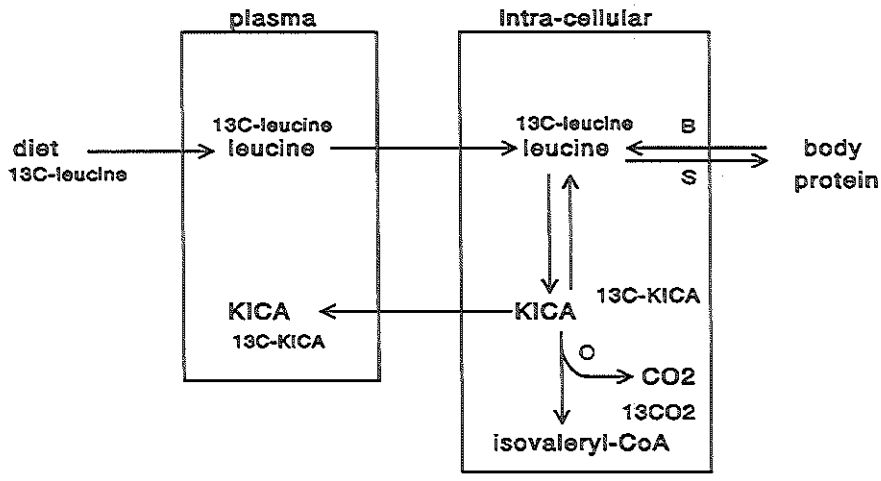
The individual amino acid kinetics can also be measured. Amino acids can leave the pool *via* incorporation into proteins or *via* oxidation as is shown in **Figure 1.9.** The measurement of oxidation however necessitates the use of a carbon-labelled tracer to be able to measure the $^{13}CO_2$ excretion. Infusion of amino acids and release of amino acids from proteins (protein breakdown) are ways of entering the pool.

Measurement and calculation of amino acid oxidation

The percentage of an amino acid, *e.g.* leucine, leaving the plasma pool, that is oxidized can be calculated from the $^{13}CO_2$ excretion. Both the enrichment in expired CO_2 and the production rate of CO_2 should be known. In preterm infants this is only possible by means of indirect calorimetry. However, the indirect calorimeter present in our unit allows only studies of stable infants, since it is a completely closed circuit system [31].

No access to the infant is possible without disturbing the measurement. It is therefore not possible to measure *e.g.* ventilated infants. We have developed an alternative method enabling the calculation of leucine oxidation in ventilated patients [32]. To measure the leucine oxidation rate with the use of a continuous infusion of [1-^{13}C]leucine, usually, a correction factor is used to compensate for the losses of labelled carbon within the bicarbonate pool.

Fig 1.9 Model of leucine metabolism.



This correction factor can be determined beforehand in a separate study or calculated from the energy intake [33]. The equation used for the oxidation is:

$$leucine\ oxidation = \frac{VCO_2(L) \times IE_{CO_2}(L)}{I(L) \times c} \times leucine\ turnover$$

$VCO_2(L)$ is the rate of CO_2 excretion in $\mu mol.kg^{-1}.min^{-1}$ during the [1-^{13}C]leucine infusion, $IE_{CO2}(L)$ is the isotopic enrichment of expired CO_2 at plateau, I(L) is the [1-^{13}C]leucine infusion rate in $\mu mol\ ^{13}C.kg^{-1}.min^{-1}$ and c is the correction factor.

A more accurate method is to measure the bicarbonate retention for each infant individually. The correction factor is determined using the following equation:

$$c = \frac{VCO_2(B) \times IE_{CO_2}(B)}{I(B)}$$

$VCO_2(B)$ is the rate of CO_2 excretion in $\mu mol.kg^{-1}.min^{-1}$ during the $NaH^{13}CO_3$ infusion, $IE_{CO2}(B)$ is the isotopic enrichment of expired air at plateau during the $NaH^{13}CO_3$ infusion and I(B) is the $NaH^{13}CO_3$ infusion rate in $\mu mol.kg^{-1}.min^{-1}$. Combining these equations leads to:

$$leucine\ oxidation = \frac{VCO_2(L) \times IE_{CO_2}(L)}{I(L) \times \frac{VCO_2(B) \times IE_{CO_2}(B)}{I(B)}} \times leucine\ turnover$$

Since the $NaH^{13}CO_3$ infusion is immediately followed by the [1-^{13}C]leucine infusion and no changes are made in the intake of the infants, we can assume that VCO_2 (L) = VCO_2 (B). Removing the term VCO_2 from both numerator and denominator, the following equation can be derived:

$$leucine\ oxidation = \frac{IE_{CO_2}(L) \times I(B)}{I(L) \times IE_{CO_2}(B)} \times leucine\ turnover$$

This equation shows that, in case of a constant CO_2 production over a few hours, it is not necessary to measure VCO_2. This enables us to study leucine oxidation in patients where the CO_2 production can not be measured.

HORMONAL REGULATION OF NITROGEN METABOLISM

Several reviews exist on the hormonal control of nitrogen metabolism [34-37]. The mechanism of the hormonal control of nitrogen metabolism seems to be species-dependent. For instance in rats, insulin clearly promotes protein accretion by an increase in protein synthesis, while in humans a higher protein accretion is obtained by lowering protein breakdown. Some studies in humans even find a decrease in protein synthesis following insulin administration. A brief overview of the main regulatory hormones and their effect on nitrogen metabolism in animals and humans will be presented here.

Insulin

Most studies on the long term effect of insulin deficiency on muscle protein metabolism have been performed in diabetic animals. An inhibition of protein synthesis has been found *in vitro* and *in vivo* in these animals [38-40]. Protein breakdown is initially, after the induction of diabetes, increased [38,39], but when the animals slowly start to grow, a decrease in muscle proteolysis is noticed [40,41]. The effect of feeding on muscle protein synthesis seems to require insulin, since starved rats did not respond to refeeding in the presence of anti-insulin serum [42,43]. Furthermore, there seems to be a synergetic effect of feeding and insulin, because insulin concentration needed to be higher in postabsorptive rats to induce protein synthesis as compared to insulin concentrations in fed animals [44]. Fetal hyperinsulinemia in lambs is associated with increased protein synthesis, together with an increase in glucose utilization [45]. Recent studies show that insulin regulates the capacity of both overall protein synthesis as well as the capacity for the synthesis of specific proteins in rat hepatocytes [46].

In man, the effect of insulin is anabolic as well, but predominantly by decreasing protein breakdown, although recently a decrease in protein synthesis has also been found following insulin infusion in diabetic patients [47]. The decrease in protein breakdown was however larger than the decrease in protein synthesis, resulting in net protein deposition [47]. Fukagawa *et al* investigated the effect of different plasma insulin concentrations on protein metabolism [48]. At five different rates of insulin infusion, they found a dose-dependent inhibitory effect on protein breakdown, while euglycemia was maintained by a variable glucose infusion. This meant that different rates of glucose had to be infused. Glucose alone however might

also exert a similar effect on protein breakdown [49]. Also Denne *et al* have found suppression of proteolysis in human adults by insulin, especially in the skeletal muscle, even at low levels of amino acids [50]. At normal plasma levels of amino acids the effects on proteolysis are more enhanced, while protein synthesis and oxidation are decreased to a lesser extent [51]. In contrast, insulin seems to promote protein synthesis and lowers leucine oxidation in septic patients [52].

Glucagon

Glucagon seems to exert a catabolic effect by two means in animals; firstly by a stimulation of gluconeogenesis in the liver and secondly *via* a depression of muscle protein synthesis. Gluconeogenic amino acids are depleted by glucagon resulting in a proteolytic effect [53]. Infusion of glucagon into growing rats depresses muscle protein synthesis, an effect which is counteracted by insulin [54,55].

In humans however, no effect on leucine, lysine and alanine flux, or *de novo* alanine synthesis was noted when peripheral insulin levels were kept constant [56]. There might be an indirect effect of glucagon on protein metabolism *via* ketone bodies, although the results described are controversial. Glucagon is a potent stimulator of hepatic ketogenesis [57]. β-Hydroxybutyrate has been shown to decrease leucine oxidation and to promote muscle protein synthesis in postabsorptive humans [58]. Studies of Felig *et al.* have shown a sparing effect on protein kinetics as well [59,60]. Miles *et al.*, however, showed that an infusion of ketone bodies does not decrease overall body proteolysis [61].

Growth hormone

Hypophysectomy in rats results in diminished growth, while growth hormone administration has a stimulatory effect on protein synthesis in lambs, mice and rats [62-64]. Protein degradation remained unchanged or tended to increase. Also in septic rats an increase in nitrogen retention was seen [65].

The anabolic effects of human growth hormone have been documented in normal volunteers. Growth hormone acts mainly on protein synthesis [66-68]. In man, growth hormone seems to counteract the catabolic effects of corticosteroids [69,70] and lowers protein oxidation [71]. In specific disorders such as growth hormone deficiency, human growth hormone decreases urea synthesis [72], and increases protein breakdown, but less so than synthesis, resulting in a higher

nitrogen retention [73].

Isulin-like Growth Factor I and II (IGFs)

IGF-I and IGF-II are single chain polypeptides and they are synthesized *de novo* in a variety of tissues. The plasma levels vary with age and increase during the last two month of fetal life. IGF-II levels are four to seven times those of IGF-I, but both levels are still very low compared with those in adolescents and adults [74]. Transcription and release is under strict growth hormone control [75]. The protein-sparing effects of growth hormone are believed to be in part mediated via the production of IGF-I. Administration of recombinant human IGF-I has been shown to restore growth in hypophysectomized rats [76] and to have a protein-sparing effect in fasted rats [77]. The fractional synthetic rate of muscle and liver protein increases in lambs following IGF-I administration [78]. In contrast, IGF-I inhibited hepatic protein metabolism, tending to decrease synthesis rates in dwarf mice [79]. Again this might indicate a species-dependent mechanism of action. Growth hormone has an anabolic effect on hepatic protein metabolism, suggesting that there might be also specific differential effects of IGF-1 and growth hormone on protein metabolism. Administration of IGF-I to patients with Laron-type dwarfism who are unable to generate endogenous IGF-I despite high growth hormone levels, results in a rapid stimulation of linear growth, a similar effect as has been reported after growth hormone administration to growth hormone deficient patients [80].

Glucocorticoids

Glucocortocoids have a profound effect on muscle. The overproduction of adrenal steroids in Cushing's syndrome or the high dosages of dexamethasone that are often administered clinically can cause growth inhibition in the young and a marked wasting of muscle in the adult. The action of dexamethasone is extensively discussed in chapter 7.

Testosterone has an anabolic effect as is shown in lambs, probably expressed through reduction of muscle protein degradation, since nitrogen retention improved, but no differences were shown in protein synthesis [81]. The effect of testosterone on protein metabolism is however very complex and depending on many factors as sex and age of the animals. In young female rats a stimulation of muscle growth has been observed [82], while no effect of testosteron was seen in male animals [83,84].

Others

The effect of thyroid hormones has been studied in rats. Thyroid hormones stimulate albumin synthesis [85], while muscle protein breakdown and synthesis are both normal after treatment with T_3 or thyroxine of hypophysectomized and thyroidectomized rats [39,86]. An important clinical feature of thyroid hormone action is that thyroid hormones have opposite effects on body growth when administered in high and low doses. Physiological levels of T_3 are essential for normal growth of muscle and other tissues, but excessive amounts, as in thyrotoxicosis, lead to a general loss of body weight and severe muscle wasting. Physiological levels stimulate protein synthesis more than protein breakdown, but high levels stimulate protein breakdown more than protein synthesis, resulting in muscle wasting [87,88]. The mechanism for this different dose-response relationship is still unclear.

A decrease in plasma amino acid levels is noticed with raising epinephrine levels, but the effect of epinephrine on protein metabolism seems to be not very important [89-91].

Interleukin 1 acts as a stimulator of muscle protein breakdown in vitro, while it also stimulates prostaglandin E_2 which promotes protein breakdown in muscle [92,93].

NITROGEN KINETICS IN CONTROLLED NUTRITIONAL STUDIES IN NEONATES

The increasing rate of survival of very low birth weight infants has challenged us to find ways of meeting their nutritional requirements. These might differ substantially from those of the healthy term infant and older children. The differences are based upon many metabolic enzyme immaturities and also upon the high requirements needed for the very rapid growth that the nutritionist would like the preterm infant to have.

Protein requirement and metabolism has been an area of great interest since accretion of body protein is essential for growth. Because of differences in the clinical condition of the patients and the different handling of the nutrients entering the body a subdivision has been made in orally and parenterally fed infants. Both will be discussed below.

Orally fed newborn infant

The importance of protein intake on weight gain has been shown by Heird and Kashyap [94]. Weight gain of infants fed 120 kcal.kg^{-1}.d^{-1} and 2.8 g protein was 16 g.kg^{-1}.d^{-1}, whereas infants fed 3.8 g protein gained weight at a mean of 19 g.kg^{-1}.d^{-1}. At a different level of energy intake (140 kcal.kg^{-1}.d^{-1}), but with a similar intake of 3.9 g protein.kg^{-1}.d^{-1}, weight was gained at a mean of 21.5 g.kg^{-1}.d^{-1} [95]. Thus an additional effect of energy intake seems to exist on protein utilization and, hence, growth. This is not remarkable since protein synthesis is an energy consuming process. Catzeflis *et al.* have calculated that the synthesis of 1 gram protein requires 2 kcal [96], whereas Moore calculated, theoretically, 1 kcal per gram protein synthesized [97]. The release of amino acids from proteins might be an energy consuming process as well [98]. The amount of energy provided to the infants could thus limit protein turnover.

Several studies have been performed to gain insight in the nitrogen metabolism of orally fed infants. Picou and Taylor-Roberts were the first in 1969, measuring protein synthesis and breakdown rates in 10-20 month old infants recovering from malnutrition [99]. Nicholson was the first to study protein synthesis in premature infants in 1970 [100]. The average rate of protein synthesis he measured was 12.7-15.4 g.kg^{-1}.d^{-1}, in three orally fed infants using [^{15}N]glycine as a tracer. **Table 1.3.** lists the results of most of the published studies performed in orally fed preterm neonates in chronological order. Around 150 orally fed preterm infants have been studied. Protein synthesis rates range from estimates of 5 to 26 g.kg^{-1}.d^{-1}. According to the amounts of energy needed to synthesize protein mentioned above, the total energy requirements just for protein synthesis should range from 5 to 52 kcal.kg^{-1}.d^{-1}. This calculation does not include the energy requirement for protein breakdown. From this point of view it seems that some of the reported protein synthesis rates are very high and are due to methodological errors. Many variables contribute to this large variation, but most of them have not yet been properly investigated. As can be seen in **Table 1.3**, most studies have been performed administering [^{15}N]glycine followed by the determination of the nitrogen enrichment in urinary urea. In particular the definition and timing of the urinary plateau are confounding factors. Assuming a plateau, while in fact the enrichment is still rising, will underestimate the true ^{15}N-enrichment at a plateau and inversely overestimate protein turnover, breakdown and synthesis rates. Some of the methodological errors involved in the [^{15}N]glycine model are discussed in chapter 3.

Rates of whole-body protein synthesis in adults, measured with [1-^{13}C]leucine, are in the order of 3-4 g.kg^{-1}.d^{-1} with no protein accretion, whereas term infants synthesize 5-6 g protein.kg^{-1}.d^{-1} , with a protein accretion of 1.2 g.kg^{-1}.d^{-1} [101]. The rates of protein synthesis show a clear decrease as postconceptional age increases.

Not only the choice of the tracer and the postconceptional age are important variables contributing to the wide range of whole-body protein synthesis, the effect of different diets might also be important. Pencharz *et al.* found higher rates of whole-body protein turnover in preterms receiving human milk compared to formula fed infants [102]. However, human milk contains a significant amount of urea, which can attribute to the urea pool. The finding of Pencharz *et al.* is based on urea enrichment following [^{15}N]glycine administration. The dietary urea could be diluting the labelled urea, resulting in apparently higher turnover rates in human milk fed preterms.

Enteral provision of nutrients appears to have a role in the growth and development of visceral organs [103,104]. It is therefore likely that the route of feeding has an influence on protein turnover in the gastrointestinal tract. In 1986, Duffy and Pencharz reported a rise of 40% in whole-body protein turnover, synthesis and breakdown rates in orally fed infants compared to intravenously fed infants, as measured by urea enrichment following [^{15}N]glycine administration [105]. Comparing individual amino acid turnovers, Wykes *et al.* found higher glycine turnover rates, unaffected leucine turnover rates and lower phenylalanine turnover rates in orally fed infants [106]. It is however difficult to interpret these data, since phenylalanine intake was twice as high in the parenterally fed infants compared to the orally fed infants. The phenylalanine turnover rate was also highly affected by the intake.

Although the route of feeding itself might exert an effect on amino acid turnover rates, the route of tracer administration is also important. Intragastric administration of tracer may result in fewer tracer molecules reaching the systemic circulation because of significant first pass removal by splanchnic tissues. This will result in a lower isotope steady state, and therefore in a higher estimated turnover. This issue has been a matter of investigation in three recent studies measuring leucine kinetics. The oxidation of leucine in the splanchnic area is not a cause of significant loss of tracer [107]. Almost all leucine taken up by the splanchnic area is released as its keto-analogue (KICA) in adults [108,109], but in very-low-birth weight infants there might be some loss of label [110]. KICA is the product of

Table 1.3. Whole-body protein synthesis and breakdown rates of orally fed preterm infants.

reference	year of public.	infants studied	postnatal age (d)	weight gain g/(kg.d)	protein intake g/(kg.d)	energy intake kcal/(kg.d)	tracer	method	S g/(kg.d)	B g/(kg.d)
100	1970	3	46	12	?	?	[^{15}N]glycine	urea	13	
								ammonia	15	
113	1977	8			3.3	136	[^{15}N]glycine	urea	26	24
114	1981	20	19	18	5.1	138	[^{15}N]glycine	urea	17	13
		20	23		4.3	156			16	12
115	1981	3	9-32	16	2.8	136	[^{15}N]glycine	ammonia	11	9
116	1983	8					[^{15}N]glycine		8	6
117	1984	7	22	15	2.9	129	[1-^{13}C]leucine	leucine	11	10
96	1985	10	26	15	3.0	114	[^{15}N]glycine	ammonia	6	4
								urea[1]	11[1]	9[1]
118	1987	4	23	17	2.6	125	[^{15}N]mixture	urine	11	10
			26	16	2.6	125	[^{15}N]glycine		16	14
			20	13	2.6	139	[^{15}N]yeast		10	8
119	1988	19	19	18	3.0[2]	110[2]	[^{15}N]glycine	urea	9	7
120	1989	11	26	16	2.7	125	[^{15}N]glycine	ammonia	14/13	11/13
							[^{15}N]yeast		20/13	18/13
121	1990	7	30	17	3.0/3.8	?	[1-^{13}C]leucine	leucine/CO_2	6/8	4/6
122	1991	14	4	-3	1.8	80	[1-^{13}C]leucine	leucine	16	16
123	1992	9	18	11	2.5	130	[^{15}N]glycine	urinary	15/8	13/6
							[^{15}N]yeast	N/NH_3	6/17	7/13
							[^{15}N]leucine		9/14	4/15
124	1992	9	8		2.0	68	[1-^{13}C]leucine	leucine/CO_2	8	7

[1] based upon 5 patients, the other 5 failed to have enrichment in urea. [2] metabolizable energy and protein intake. [3] except for one infant who received breast milk.

intracellular transamination of leucine and its plasma enrichment is very close to intracellular [1-^{13}C]leucine enrichment [111,112]. The enrichment of KICA thus reflects the intracellular enrichment of leucine. Both proteolysis and protein synthesis are processes occurring intracellularly and it seems therefore that the determination of enrichment of KICA following [1-^{13}C]leucine administration (reciprocal pool model) will give the proper enrichment for calculations of protein kinetics. The route of administration of the tracer is not a confounding variable in parenterally fed preterm infants, since the tracer follows exactly the same pathway as the nutrients.

Parenterally fed infant

Preterm infants have limited energy reserves and are often unable to tolerate oral feeding in amounts necessary to meet their needs. Parenteral nutrition is therefore given for some period to almost all very-low-birth weight infants. Parenteral nutrition for the preterm infant should theoretically simulate intra-uterine feeding. It can however be difficult in practice to achieve sufficient levels of intake to mimic intra-uterine growth. Morbidity in the infant (respiratory distress, patent ductus arteriosus, renal disease) may limit fluid intake and thus restrict energy and nutrient intake, which in its turn limits nitrogen retention and thus growth.

There is no universal agreement as to the timing of the introduction of amino acids to sick infants who need to be fed parenterally. In many cases amino acid feeding is delayed for 3-4 days postnatally [11]. It is clear that this has implications for the deposition of lean tissue. Concerns about protein hydrolysates has led to this delay in the introduction of an amino acid source, because of various metabolic complications including hyperammonemia and acidosis.

Data on protein synthesis and breakdown in parenterally fed preterm infants are relatively scarce. **Table 1.4** summarizes protein synthesis and breakdown rates currently available from literature in 81 parenterally fed preterm infants. Protein synthesis and breakdown rates are within a relatively small range, considering the difference in amino acid and energy intake. Clearly, the effect of energy and amino acids can be seen, with increasing rates of protein synthesis with increasing intakes. The data from Duffy *et al.* [125], obtained by using [^{15}N]glycine, are in close agreement with the other studies, using [1-^{13}C]leucine as a tracer.

There are no generally accepted guidelines on the amino acid intakes for parenterally fed preterm infants from for instance the ESPGAN (European Society of Paediatric Gastroenterology).

Table 1.4. Whole-body protein synthesis and breakdown rates of preterm infants on parenteral nutrition.

ref.	year of public.	infants studied	postnatal age (d)	clinical condition	amino acid intake g/(kg.d)	energy intake kcal/(kg.d)	tracer	method	S g/(kg.d)	B g/(kg.d)
126	1991	19	2	ventilated	0	35	[1-^{13}C]leucine	primary	5.2	5.9
127	1993	7	3	ventilated	0	35	[1-^{13}C]leucine	reciprocal	5.0	6.7
127	1993	5	3	ventilated	1.6	54	[1-^{13}C]leucine	reciprocal	6.9	7.4
128	1992	20	3-7	ventilated	0.9 - 2.6	31 - 88	[1-^{13}C]leucine	primary	6.0 - 9.3	6.0 - 7.5
		6	10		2.7?	88?			10.5	8.9
125	1981	8	7	"ill"	2.7	90	[^{15}N]glycine	Urea	8.8	7.6
		8			2.5	66			7.5	6.7
		8			2.9	96			8.9	7.1
		8			2.6	70			6.8	5.3

Fetal accretion rates and protein balance and specific amino acid turnover studies could provide very useful information for total amino acid requirements and for specific amino acid requirements.

AIMS OF THIS THESIS

The underlying theme of this work has been to try to understand the protein metabolism in the premature human infant, and its response to perturbations including disease and diet. Since the early work of Schoenheimer *et al.* it has been recognized that growth takes place as a result of protein synthesis and protein breakdown [16]. With the understanding of protein metabolism itself and the response of protein metabolism to diseases and diet, an improved therapy (treatment as well as nutrition) is possible, leading to an improved growth.

The following questions were raised:

- Is it possible to improve nitrogen retention during the first few postnatal days *via* amino acid supplementation, despite a very low energy intake? And if so, is protein synthesis increased, protein breakdown lowered or are both mechanisms acting simultaneously? Is this effect still measurable after one week?
- Two tracers have been used frequently to measure whole-body protein kinetics; [^{15}N]glycine and [1-^{13}C]leucine. Are they comparable?
- What is the effect on protein metabolism of administration of amino acid solutions with a different chemical compostion to preterm infants?
- Corticosteroids are known to cause protein wasting in adults. What is the effect of dexamethasone, given as therapy for bronchopulmonary dysplasia, on protein kinetics in preterm infants?

The sequence of the studies presented in this thesis reflects in some manner the chronological order in which the studies have taken place.

The influence on nitrogen kinetics of commencing with an amino acid solution on day two of life is compared to commencing on day four of life in **chapter 2.** This study started in 1989. At that time, the policy of many neonatal intensive care units in the Netherlands and abroad was reluctance to give early

nutritional support to premature infants. To trace nitrogen metabolism to get information on whole-body protein synthesis and breakdown rates, we used [^{15}N]glycine.

However there were concerns about the validity of [^{15}N]glycine to monitor nitrogen kinetics. At the end of 1989 we started to compare the use of [^{15}N]glycine with the use of [1-^{13}C]leucine to measure protein metabolism. This study was part of a larger study defining optimal energy and protein requirements for orally fed growing premature infants. The comparison of the two tracers is described in **chapter 3.**

The other studies described in this thesis were all performed at the end of 1991 and in 1992. Protein kinetics were measured with [1-^{13}C]leucine as a tracer. A follow-up of the study described in chapter 2 is presented in **chapter 4.** A comparison is made regarding nitrogen metabolism and plasma amino acid levels between infants receiving amino acids directly from birth onwards

The quality of amino acid solutions administered to preterm infants is investigated by means of plasma amino acid levels measured after six days of total parenteral nutrition. This study is described in **chapter 5.** Special attention is given to two, for premature infants essential, amino acids cysteine and tyrosine.

In **chapter 6** the influence of two variables on protein kinetics is measured: Firstly, the influence of different amino acid solutions on protein kinetics was measured in preterm infants receiving the amino acid solution from day 2 of life onwards. Measurements were performed at one week of age. Secondly, the effect of commencing amino acid supplementation from birth onwards on protein kinetics was compared to starting at day 2. Again measurements were made at one week of age.

Finally, in **chapter 7,** the effect of dexamethasone therapy given to infants with bronchopulmonary dysplasia on nitrogen metabolism is shown.

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CHAPTER 2

EFFECTS OF EARLY AMINO ACID ADMINISTRATION DURING TOTAL PARENTERAL NUTRITION ON PROTEIN METABOLISM IN PRETERM INFANTS[24,25]

SUMMARY

1. We investigated the effects of starting amino acid administration on postnatal day 2 on protein turnover and nitrogen balance in appropriate-for-gestational-age, very-low-birth-weight infants. Eighteen infants were divided into two groups. Group A received from day 2 onwards an amino acid solution, whereas group B started on this solution after day 4. Both groups were exclusively parenterally fed, 200 $kJ.kg^{-1}.d^{-1}$ on postnatal days 3 and 4. Group A (birth weight 1.5 $\pm$ 0.3 kg) received 4.6 g of glucose, 1.9 g of fat and 2.3 g of amino acids day^{-1} kg^{-1} body weight. Group B (birth weight 1.4 $\pm$ 0.2 kg) received 7.0 g of glucose and 1.9 g of fat day^{-1} kg^{-1} body weight.

2. At postnatal day 3, a primed constant infusion of 3 mg [^{15}N]glycine day^{-1} kg^{-1} was given. Protein flux, protein synthesis and protein breakdown were calculated from the ^{15}N enrichment in urinary ammonia. In five out of nine infants in group B no plateau of ^{15}N enrichment in urinary urea could be detected, whereas in group A two out of nine infants did not reach a plateau. For this reason we did not use the end product urea for our calculations.

3. The administration of the amino acids resulted in a higher protein flux (6.9 $\pm$ 1.5 $g.kg^{-1}.d^{-1}$) A *versus* 5.2 $\pm$ 0.9 $g.kg^{-1}d^{-1}$ B) and a higher protein synthesis (6.0 $\pm$ 1.4 $g.kg^{-1}d^{-1}$ A *versus* 4.6 $\pm$ 0.8 $g.kg^{-1}d^{-1}$ B). There was no statistically significant difference in protein breakdown. The administration of amino acids reversed a negative protein balance (-0.6 $\pm$ 0.2 $g.kg^{-1}d^{-1}$) into a positive one (1.4 $\pm$ 0.2 $g.kg^{-1}.d^{-1}$). No adverse affects of the amino acid infusion were seen.

4. We conclude that early introduction of amino acids has, even at this relatively low energy intake of 200 $kJ.kg^{-1}.day^{-1}$ a positive effect on protein balance by increasing protein synthesis.

INTRODUCTION

Parenteral feeding is given during the first few days of life to infants who are thought not to tolerate oral feeding because of respiratory distress, asphyxia, gastrointestinal disorders or immaturity [1,2] or to prevent necrotizing enterocolitis [3]. Although a consensus has been reached that the aim of nutrition should be to provide at least an intake similar to the intrauterine intake, the time at which amino acids should be introduced is still a matter of debate. Several authors recommend starting within 24 h of birth, whereas others advocate waiting for at least 4 days

after birth [4-6].

Protein is continuously synthesized from and broken down into amino acids. This protein turnover can be estimated by using different labelled amino acids, such as [^{15}N]glycine and [^{13}C]-leucine, as tracers [7,8]. Under defined circumstances, comparable estimates of protein turnover have been obtained using the different amino acids simultaneously [9,10]. No studies have evaluated the effect of administering amino acids from the first post-natal day onwards on protein kinetics in preterm infants.

In the present study we report the effects of the early introduction of amino acids on nitrogen balance and protein turnover, measured by using ^{15}N labelled glycine as a tracer.

PATIENTS

The subjects of the study were admitted to our neonatal intensive care unit. Eighteen preterm, appropriate-for-gestational-age (AGA), infants entered the study. AGA was defined as birth weight between the 3rd and 97th percentile for gestation (Usher and McLean [11]). Gestational age was assessed from the date of the last menstrual period, ultrasound data and Ballard score [12]. All infants were clinically stable at the time of the study, with normal renal, liver and gastrointestinal functions. The infants were randomly divided into two groups of nine, and were all exclusively parenterally fed. On the first post-natal day both groups received glucose only. From day 2 onwards, one group (A) received glucose, fat (10% (w/v) Intralipid, Kabi Vitrum, Sweden) and amino acids (10% (w/v) Aminovenös, Fresenius, Bad Homburg, Germany); the other group (B) received glucose and fat only.

Table 2.1 Clinical characteristics of the infants. Values are mean ± SD.

	Group A (with amino acid administration)	Group B (without amino acid administration)
No. of infants	9	9
Birth weight (kg)	1.51 ± 0.3	1.40 ± 0.2
Gestational age (wk)	30.7 ± 2.4	31.0 ± 1.4

The intake was made isocaloric for both groups by administering more glucose to the patients in group B to meet the protein energy given in group A. Amino acid administration was constant for at least 12 h before measurements were made. The clinical characteristics of the infants are summarized in Table 2.1. During the study six patients in group A were ventilated, four infants in group B. In both groups six patients were treated for hyperbilirubinaemia with phototherapy. During the study, all infants received antibiotic treatment, because of the suspicion of septicaemia; however, all bloodcultures were found to be negative. Table 2.2 describes the intake of the infants during the study.

MATERIALS AND METHODS

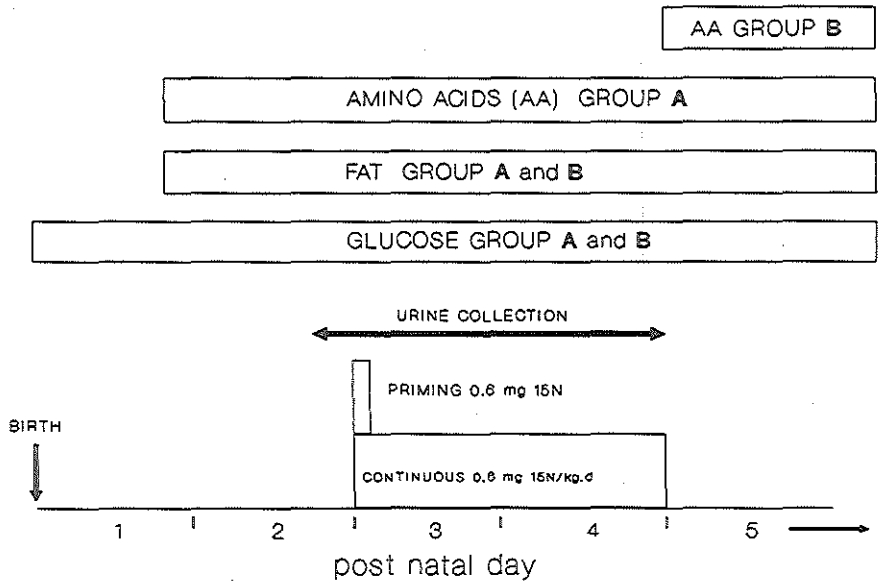
Informed consent from one or both of the parents was obtained before the study. The study was approved by the Ethical Committee of our hospital. All studies were carried out in a standard incubator on the Neonatal Intensive Care Unit of our hospital and did not interfere with the normal routine on the ward.

Stable isotope study. [^{15}N]glycine (99 atom% excess) was purchased from MSD, Montreal, Canada. The glycine was dissolved in distilled water and the solution was made sterile by autoclavation. At post-natal days 3 and 4, a primed constant parenteral infusion of [^{15}N]glycine was given. An outline of the study is given in Fig 2.1. The priming dose was 3 mg [^{15}N]glycine/kg. A continuous intravenous infusion,

Table 2.2 Intake during the study in the two groups of infants. Values are mean±SD.

	group A	group B
Glucose ($g.kg^{-1}.d^{-1}$)	4.6 ± 1.5	7.0 ± 1.2
Fat ($g.kg^{-1}.d^{-1}$)	1.9 ± 0.4	1.9 ± 0.7
Amino acids		
$g.kg^{-1}.d^{-1}$	2.3 ± 0.2	-
mg $N.kg^{-1}.d^{-1}$	368 ± 32	-
mg of $glycine.kg^{-1}.d^{-1}$	98 ± 8	-
Energy ($kJ.kg^{-1}.d^{-1}$)	200 ± 29	197 ± 34

Fig. 2.1 Outline of the study. Abbreviation: AA, amino acids.



3 mg [^{15}N]glycine.kg^{-1}.day^{-1}, was given for 48 h, together with the parenteral nutrition.

Before the administration of [^{15}N]glycine we collected urine to determine the natural background of ^{15}N. During the study, (postnatal days 3 and 4), urine was collected in 3 hourly periods using adhesive bags. Immediately after each period the urine was stored at -20°C.

Analysis of the urine was as follows. Using Lips-Conway micro-diffusion cells, ammonia was trapped in sulphuric acid by incubation of the urine at pH 12. In the resulting ammonia-free urine, urea was converted to ammonia by adding urease. This ammonia was collected in the same way [13].

The ammonia-nitrogen was led into an automatic nitrogen analyser (ANA 1400; Carlo Erba, Milan, Italy). The nitrogen gas leaving the nitrogen analyser was led directly into an Isotope Ratio Mass Spectrometer (SIRA 10, VG, Winsford, Cheshire, England), for measurment of the ^{15}N enrichment, expressed as Atom%.

Protein flux was calculated, using the method described by Picou and Taylor-Roberts, from the plateau enrichment of [^{15}N]-ammonia and [^{15}N]-urea [14]. The

assumptions and calculations are described briefly. The method assumes that there is a single metabolic pool of nitrogen or amino acids from which protein is synthesized and from which urea and ammonia are formed. In steady state the amount of nitrogen leaving this pool (synthesis and excretion in urine) is the same as the amount of nitrogen entering the pool (protein breakdown and exogenous intake). Protein turnover or flux is the amount nitrogen leaving or entering the pool. In equation:

$$Q = S + E = B + I$$

where Q is flux or turnover, S is synthesis, E is excretion, B is breakdown and I is intake. In the stochastic model, where [^{15}N]glycine is administered at a constant rate, flux can be measured if there is a constant ratio of ^{15}N to ^{14}N measured in urinary urea or ammonia. This plateau is reached when there is isotopic equilibrium, i.e. the enrichment in the metabolic nitrogen or amino acid pool is the same as the end products formed from that pool. In equation:

$$Q = \frac{d}{IE}$$

where d is the rate of administered isotopic nitrogen and IE is the isotopic enrichment in the end product at plateau.

Plateau values were determined by visual inspection and confirmed by regression analysis.

Nitrogen balance study. A nitrogen balance was performed during 48 h, simultaneously with the collection of urine for the stable isotope study. The total urinary nitrogen excretion was determined in triplicate using the automatic nitrogen analyser. The balance was calculated by subtracting the total urinary nitrogen excretion from the nitrogen intake. During the total parenteral nutrition, nitrogen loss in the stools was assumed to be negligible. Protein values were calculated by multiplying the nitrogen value by a factor of 6.25.

Statistics. Data are presented as mean$\pm$SD. For statistical analysis, the Mann-Whitney U-test was used. A P value of <0.05 was considered to be statistically significant.

Table 2.3 The individual time when plateau was reached, with the corresponding atom percent excess (ΔIE) in urinary ammonia and urea.

Subject	Ammonia		Urea	
	time (h)	*ΔIE* (atom% excess)	time (h)	*ΔIE* (atom% excess)
Group A				
1	3	0.0545	3	0.0606
2	12	0.0451	12	0.0492
3	18	0.0516	15	0.0374
4	12	0.0600	24	0.0396
5	9	0.0349	21	0.0304
6	24	0.0550	30	0.0366
7	12	0.0627	-	0.0303†
8	9	0.0689	-	0.0492†
9	9	0.0538	12	0.0581
Mean±SD		0.0541±0.099		
Group B				
10	12	0.0541	-	0.0721†
11	18	0.0597	-	0.0465†
12	24	0.0909	-	0.0303†
13	18	0.0584	-	0.0241†
14	15	0.0759	24	0.0803
15	15	0.0669	36	0.0565
16	12	0.0881	39	0.0347
17	9	0.0682	36	0.0294
18	18	0.0781	-	0.0574†
Mean±SD		0.0711±0.0130		

†No plateau was reached; the highest value reached is given instead of the plateau value.

RESULTS

We found no significant differences in birth weight or gestational age between both groups (Table 2.1). The caloric intake, as well as the fat intake, during the study, showed no significant difference either.

A plateau in [^{15}N]-ammonia excretion was found within 27 h after the start of the infusion of [^{15}N]glycine in all studies. Using [^{15}N]-urea as an end-product, a plateau in ^{15}N excretion could not be detected in a majority of patients in group B (five out of nine)and in a minority of patients in group A (two out of nine). Details of the isotopic enrichment of urinary urea and ammonnia and the time when the plateau was achieved are given in Table 2.3. Whenever no plateau was reached, the highest value of enrichment is given. Because no plateau in [^{15}N]-urea excretion was reached in seven patients no calculations could be based on [^{15}N]-urea enrichment. All results concerning protein turnover are therefore based on [^{15}N]-ammonia as an end-product.

A significantly higher protein flux in group A compared with group B was found (6.9 $\pm$ 1.5 g.kg^{-1}d^{-1} (A) *versus* 5.2 $\pm$ 0.9 (B), $P < 0.05$), Table 2.4). Protein breakdown was not significantly different between the two groups. The administration of amino acids did result in a significantly higher protein synthesis (group A, 6.0 $\pm$ 1.4 g.kg^{-1}d^{-1}; group B, 4.6 $\pm$ 0.8 g.kg^{-1}d^{-1}, $P < 0.05$).

We found a significant ($P < 0.001$) shift, from a negative to a positive nitrogen balance (- 96 mg N.kg^{-1}.d^{-1} to + 224 mg N.kg^{-1}.day^{-1}) when comparing the two groups (Table 2.4). The plasma urea levels, measured on postnatal day 3 in four infants of both groups, were all within the normal range (group A, 3.5 $\pm$ 1.6 mmol/l; group B, 2.8 $\pm$ 0.9 mmol/l). Although we found a tendency towards a lower pH in group A at post-natal day 4, (7.24 $\pm$ 0.05 *versus* 7.29 $\pm$ 0.05 P = 0.12), no difference was found in the base excess, nor in the amount of administered bicarbonate on that day.

DISCUSSION

^{15}N-glycine has been used extensively to study protein turnover. The method is non-invasive, which is an advantage, especially in very-low-birth-weight infants. Many different ways of administering [^{15}N]glycine, i.e. oral versus parenteral, continuous versus single dose, have been described [15]. Urea or ammonia have both been used as end product, and the average of the two end products has also been used

[8,16,17]. We have chosen a primed, continuous, parenteral infusion of [^{15}N]glycine for 48 h for the following reasons. The route of administration was intravenous, because the nutrients were also given parenterally. Other ^{15}N labelled end-product studies in preterm infants have used 72 h of constant infusion to reach a plateau in [^{15}N]-urea excretion [7,8,10,18,19]. By priming with a 24 h dose of [^{15}N]glycine we hoped to achieve a steady state sooner (i.e. before 48 h) [16]. This would reduce the time of infusion and therefore the influence of recycling on the plateau height. Recycling of the label will cause a rise in the enrichment and, since flux is inversely related to enrichment, a decrease in turnover, synthesis and breakdown. Also, it was necessary to reduce the time of isotope infusion and urine collection, because we found it unethical to withhold the infants of group B from amino acid administration for more than 4 days, so a plateau had to be reached within 48 hours.

In all cases a plateau was reached in [^{15}N]-ammonia excretion within these 48 h. However, in a majority of cases in the non-protein group (five out of nine) no plateau was reached in [^{15}N]-urea excretion. In the protein group, two out of nine patients did not reach a plateau in [^{15}N]-urea excretion. Several authors have found difficulties in urea becoming enriched, mostly in preterm infants fed human milk [7,18]. Jackson *et al.* [18] explained this finding by an impaired endogenous glycine synthesis in premature infants on a low glycine intake. A low glycine intake could also account for most, but not all, cases of low urea enrichment in the study of Catzeflis *et al.* [7]. In our study, both infants in group A who did not reach a plateau had daily glycine intakes above 90 mg/kg.

Another explanation for not reaching a plateau in [^{15}N]-urea excretion could be the slow turnover and excretion rate of urea. Steffee *et al.* [20] found a lower turnover rate of the urea pool with a fall in protein intake in adults. A plateau in urinary [^{15}N]-ammonia is usually reached before a plateau in [^{15}N]-urea. This can also be seen in our study (Table 2.3). The [^{15}N]-ammonia plateau was reached within 14 h on average, whereas it took on average almost 23 h to reach a plateau in [^{15}N]-urea excretion if such a plateau was reached. This is attributed to the smaller size of the ammonia pool compared with the urea pool, but also to the higher turnover of the ammonia pool [15].

It is well-known that administration of exogenous amino acids will increase the protein turnover. [^{15}N]-Ammonia plateaus were also reached sooner in the supplemented group than in the non-protein group. Taken these arguments into account, it is not surprising that it takes longer, especially in the non-protein group, to reach a constant [^{15}N]-urea excretion than to reach a constant [^{15}N]-ammonia

Table 2.4. Protein kinetics in the two groups of infants. Values are mean ±SD.

	Group A	Group B
Flux ($g.kg^{-1}.d^{-1}$)	6.9 ± 1.5	5.2 ± 0.9†
Synthesis ($g.kg^{-1}.d^{-1}$)	6.0 ± 1.4	4.6 ± 0.8†
Breakdown ($g.kg^{-1}.d^{-1}$)	4.6 ± 1.6	5.2 ± 0.9
Protein balance ($g.kg^{-1}.d^{-1}$)	1.4 ± 0.2	-0.6 ± 0.2‡

Statistical significance: †$P<0.05$, ‡$P<0.001$ group A *versus* group B.

excretion, after a continuous infusion of [^{15}N]glycine. Since almost all infants who did not reach a plateau in [^{15}N]-urea excretion, did have a gradual increase in [^{15}N]-urea enrichment, we believe that we just did not collect urine long enough to reach a plateau in [^{15}N]-urea excretion.

The introduction of amino acids shortly after birth did not affect breakdown, but did have a positive effect on protein synthesis. This resulted in a positive nitrogen balance (224 mg $N.kg^{-1}.day^{-1}$), which has also been described by Pencharz *et al.* [10] in parenterally fed preterm infants. However, the energy and protein intake in this study were much higher than in our study (378 *versus* 200 $kJ.kg^{-1}.day^{-1}$ and 3.1 *versus* 2.4 g $protein.kg^{-1}.day^{-1}$, respectively). These higher intakes resulted in an only marginally better nitrogen balance. (273 *versus* 224 mg $N.kg^{-1}.day^{-1}$, respectively).

Saini *et al.* [5] found a negative nitrogen balance (-133 $mg.kg^{-1}.day^{-1}$) in preterm infants who were not supplemented with amino acids and received 151 $kJ.kg^{-1}.day^{-1}$ during the first 72 h of life. In a protein-supplemented group with an energy intake of 188 $kJ.kg^{-1}.day^{-1}$ and a nitrogen intake of 286 mg $N.kg^{-1}.day^{-1}$ ($\approx$1,8 g $protein.kg^{-1}.day^{-1}$) the nitrogen retention was 120 mg $N.kg^{-1}.day^{-1}$ ($\approx$ 0.75 g $protein.kg^{-1}.day^{-1}$). They did not perform a stable isotope study, so it is not known whether the positive nitrogen balance resulted from an improved protein synthesis or a diminished protein breakdown.

Zlotkin *et al.* [21] found, with an almost similar caloric intake of 209 $kJ.kg^{-1}.day^{-1}$ and a higher protein intake (3.1 $g.kg^{-1}.day^{-1}$), a slightly higher nitrogen retention (256 mg $N.kg^{-1}.day^{-1}$ $\approx$ 1.6 g $protein.kg^{-1}.day^{-1}$).

We observed no adverse effects of the early introduction of amino acids. Although there was a tendency towards a lower pH in the supplemented group, this

could not be due to metabolic acidosis, since base excess and the amount of administered bicarbonate were not different on post-natal day 4. As was determined in four patients in both groups, no difference was found in the plasma level of urea. This finding is consistent with other studies [21]. Plasma amino acid profiles in preterm infants supplemented with amino acids during the first 3 days of life, have been noted not to be very high [4,22].

In this study, we found no statistically significant effect of administering amino acids on protein breakdown. To our knowledge, no such study has been performed in preterm infants, making it difficult to compare these data. Several authors have found both effects on synthesis as well as on breakdown in adults with different protein intakes. In young men in a fed state, Motil *et al.* [23] found no effect of increasing dietary protein intake on protein breakdown, when measured by using [^{15}N]-Lysine, whereas using [1-^{13}C]leucine an increase in protein breakdown could be observed in the group with a very low protein intake (0.1 g of protein.kg^{-1}.d^{-1}). A highly speculative explanation for our finding could be that in these newborn infants there is a certain minimal level of protein breakdown which can not be altered by increasing the amino acid load. Further studies have to be performed to confirm this speculation.

We conclude that early introduction of amino acids in preterm, AGA, infants increases protein flux and promotes protein synthesis, wheras protein breakdown is not significantly affected. Moreover, a positive nitrogen balance can be reached with administration of amino acids at a caloric intake as low as 200 kJ.kg^{-1}.d^{-1}). Since no adverse effects could be observed, starting amino acid administration on post-natal day 2 can be recommended.

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CHAPTER 3

WHOLE BODY PROTEIN TURNOVER IN PRETERM AGA AND SGA INFANTS; COMPARISON OF [^{15}N]GLYCINE AND [1-^{13}C]LEUCINE ADMINISTERED SIMULTANEOUSLY.[50]

ABSTRACT

Measurements of whole-body protein turnover in preterm infants have been made using different stable isotope methods. Large variation in results has been found, which could be due to different clinical conditions and/or the use of different tracers. We studied 14 AGA and 9 SGA orally fed preterm infants using [^{15}N]glycine and [1-^{13}C]leucine simultaneously, which allowed us to make a comparison of commonly used methods to calculate whole-body protein turnover. The infants had a postnatal age of four weeks and received on average 3.2 g protein and 110 kcal.kg^{-1}.d^{-1}hole-body protein turnover was calculated from [^{15}N] enrichment in end products: urinary ammonia, urinary urea and their means following a 72 hour [^{15}N]glycine administration and from the [^{13}C] enrichment in expired CO_2 after administration of [1-^{13}C]leucine in combination with the nitrogen balance. Enrichment of α-keto-isocaproic acid following [1-^{13}C]leucine constant infusion was measured as a direct parameter of whole-body protein turnover.

Group means for whole-body protein turnover using [^{15}N]glycine or [1-^{13}C]leucine ranged from 10 - 14 g.kg^{-1}.d^{-1}, except when using the end product method that assumes a correlation between leucine oxidation and total nitrogen excretion. There was no correlation between leucine oxidation and nitrogen excretion.

We did not find a correlation between the individual turnover rates obtained via the different isotope methods. We found very low [^{15}N] enrichment of urinary urea in the majority of SGA infants. These infants also had a lower nitrogen excretion in urine and oxidized less leucine.

Although the group means of whole-body protein turnover rates for most of the methods seem to correspond well in this patient group, [^{15}N]glycine does not seem to exchange its label with the body nitrogen pool to a significant degree and is therefore not always suitable as a carrier for [^{15}N] in protein turnover studies in premature infants.

Abbreviations:

AGA	Appropriate for Gestational Age
SGA	Small for Gestational Age
α-KICA	α-Keto-isocaproic Acid

INTRODUCTION

The use of stable isotopes for measuring metabolic processes in vivo has a clear ethical advantage over radio active isotopes. The nonradioactive character allows their use in pregnancy and in infants. Schoenheimer, Ratner and Rittenberg used stable isotopes to study protein turnover as early as 1939 [1], but more extensive research has been done since the 1970s, when analytical techniques improved remarkably [2]. From then onwards, numerous protein turnover studies have been performed using stable isotopes, in adults as well as in children. ^{15}N-labelled amino acids have been favoured for many years in preterm infants because of the non-invasive character of the studies. The method allows quantification of protein metabolism by measurement of isotope enrichment in an end product of protein catabolism in urine [3-10]. [1-^{13}C]leucine, another stable isotopic labelled tracer, has been used for protein studies in preterm neonates more recently, with either the measurement of isotopic enrichment of [1-^{13}C]leucine or [^{13}C]α-keto-isocaproic acid (α-KICA) in urine [11,12] or plasma [13-17]. The different tracers give different results, i.e. for whole body protein synthesis, rates ranging from 5 $g.kg^{-1}.d^{-1}$ to 26 $g.kg^{-1}.d^{-1}$ have been reported, depending on clinical and experimental conditions [3,14]. Studies were performed comparing different tracers and amino acids in adults [18] and in term infants [19] or comparing different amino acids with a similar label [8,9,20]. However, to date there are no studies comparing the two most commonly used labelled amino acids, [^{15}N]glycine and [1-^{13}C]leucine, in preterm neonates.

The aim of the present study was to measure the protein turnover rates in orally fed, growing preterm infants using [^{15}N]glycine and [1-^{13}C]leucine simultaneously with measurement of enrichment in urinary NH_3 and urea, plasma keto-isocaproic acid and expired CO_2.

PATIENTS

Twenty-three stable, growing preterm infants were studied (Table 3.1). Small for gestational age (SGA) infants were defined as infants with a birth weight more than two standard deviations below the mean birth weight for gestation whereas appropriate for gestational age (AGA) infants had a birth weight between - 2 sd and + 2 sd according to the charts of Usher and McLean [21]. Gestational age was determined by maternal history and Ballard score [22].

Table 3.1. Clinical characteristics of the infants. Mean ± SD.

	AGA n=14	SGA n=9
Birth weight (kg)	1.2 ± 0.2	1.0 ± 0.2†
Gestational age (wk)	29 ± 1	32 ± 2‡
Age at study (d)	29 ± 8	24 ± 6
Weight at study (kg)	1.5 ± 0.2	1.2 ± 0.1‡
Weight gain ($g.kg^{-1}.d^{-1}$)	16.3 ± 3.1	17.3 ± 2.1
Energy intake ($kcal.kg^{-1}.d^{-1}$)	110 ± 10	108 ± 12
Protein intake ($g.kg^{-1}.d^{-1}$)	3.2 ± 0.1	3.2 ± 0.2

Statistical difference between AGA and SGA infants at $P < 0.05$ is indicated by †, and at $P < 0.01$ by ‡.

At the time of the study, the infants had been fed for at least 1 week a preterm formula (Nutricia, Zoetermeer, The Netherlands) and were on full enteral feedings for at least three days. As this study is part of a larger study that determines metabolic effects of different energy intakes, infants were fed either a formula containing 80 kcal per 100 ml or 67 kcal per 100 ml. The protein content of both formulas was equal, 2.2 g per 100 ml.

Informed consent from the parents was obtained in all cases and the study had the approval of the local ethics committee.

METHODS

Seventy-two-hour nitrogen balance and [^{15}N]glycine whole-body protein turnover studies were carried out by collection of urine in 3 hourly periods using adhesive bags. During this period the feeding was given in a semi-continuous mode (every 15 minutes an aliquot of formula) through a naso-gastric tube. [^{15}N]glycine (99 atom% excess ^{15}N, MSD, Montreal, Canada) was given in hourly intervals at a dosage of 3 mg [^{15}N]glycine.$kg^{-1}.d^{-1}$ through the naso-gastric tube. Analysis of [^{15}N] enrichment of urinary ammonia and urea and total nitrogen excretion was performed as previously described [10]. Visual inspection was used to determine the height of the

plateau. Nitrogen losses in the stools and through the skin were assumed to be 10% of the intake.

On the second day of the [^{15}N]glycine turnover study and the nitrogen balance, a primed constant oral infusion of [1-^{13}C]leucine (priming dose 2 mg· kg^{-1}, continuous 2 mg· kg^{-1}· h^{-1}, 99 atom% excess ^{13}C, Isotech, Miamisburg, Ohio, USA), with an additional priming of the bicarbonate pool with 6.9 μmol/kg ^{13}C-labelled sodium bicarbonate (99 atom% excess ^{13}C, Isotech, Miamisburg, Ohio, USA), was administered for 4 hours, using a Harvard infusion pump (M22, Harvard Apparatus Co Inc S. Natick, MA, USA). The formula was also administered continuously during this period. The infants were nursed in a closed circuit indirect calorimetry incubator during the [1-^{13}C]leucine turnover study. VCO_2 was measured and expired air was collected before and during the [1-^{13}C]leucine administration by passing a sample of air leaving the calorimeter through an all-glass spiral condenser, containing 10 ml of a fresh 1 M NaOH solution [23]. After liberating CO_2 by adding phosphoric acid (85%) to the solution, $^{13}CO_2/^{12}CO_2$ isotope ratio was measured on an isotope ratio/mass spectrometer (SIRA 10, VG, Winsford, Cheshire, UK).

Three blood samples were drawn, one prior to the start of the study, and two at the end of infusion of [1-^{13}C]leucine with a 15 minute interval. Within 15 minutes, plasma was stored at -70° C until further analysis. The plasma enrichment of keto-isocaproic acid was measured by gas-chromatography/mass spectrometry with electron impact and selected ion monitoring [24].

CALCULATIONS

Required assumptions and the calculations to provide whole-body protein turnover rates by methods frequently reported in the literature are briefly presented. Their relative merits will be discussed later.

There are in general two methods to calculate whole body protein turnover with stable isotope labelled tracers, either using (1) enrichment in end-products or (2) the direct measurement of the plasma enrichment of an essential amino acid. The former (1) is based upon measurement of labelled urea and ammonia as end products of protein metabolism in [^{15}N] tracer studies or upon the excretion of $^{13}CO_2$ in expiratory air that is formed at the decarboxylation step as a function of oxidation in [^{13}C] tracer studies. Direct measurement of the dilution of a tracer (2) is usually performed in plasma or urine. Leucine is an essential amino acid that is very often

used to assess protein turnover with a direct measurement in plasma [eg 13].

End products and [^{15}N]glycine.

(calculation 1) The measurement of protein turnover using ^{15}N-urea enrichment in urine.

The method relies on the concept that there is a single homogenous pool of metabolically active nitrogen in the body [1]. [^{15}N] enters this pool by means of [^{15}N] labelled glycine and is freely exchanged between other amino acids. The incorporation of nitrogen into protein and urea is assumed to arise from the same precursor pool.

Nitrogen flux (Q) through this pool is calculated by dividing the amount of administered tracer (d) by the enrichment in urea (IE_{urea}) [2].

$$Q_{urea} = \frac{d}{IE_{urea}} \qquad (1)$$

This simple model is one of the most commonly used methods for studying protein metabolism in preterm neonates.

(calculation 2) The measurement of protein turnover using ^{15}N-ammonia enrichment in urine. The model is almost identical to the one described above, the difference being the measurement of enrichment in urinary ammonia instead of urea.

$$Q_{NH_3} = \frac{d}{IE_{NH_3}} \qquad (2)$$

(calculation 3) The single pool model, as used in calculations 1 and 2, was questioned because of the differences found in enrichment of urea and ammonia [25,26]. The next simplest model with the use of end-products is the concept of two compartments, from which either urea or ammonia arises. Different mathematical estimates of protein turnover can be obtained using different assumptions regarding flux rates through the compartments and partitioning of the tracer. Assuming an equal partitioning of the tracer dose between the two metabolic compartments the nitrogen flux equals the arithmetic average of the values for flux obtained individually.

$$Q_{aa} = \frac{Q_{ammonia} + Q_{urea}}{2} \quad (3)$$

(calculation 4) An alternative method to calculate whole-body protein turnover rates based on both urea and ammonia enrichment is by using the harmonic mean. The basic assumption in this calculation is that the nitrogen flux through the pool from which urea is derived is similar to the nitrogen flux through the pool from which ammonia is derived, in equation:

$$Q_{ha} = \frac{2}{(1/Q_{ammonia} + 1/Q_{urea})} \quad (4)$$

An isotopic ratio mass spectrometer (IRMS) is required to measure the [^{15}N] enrichment in urinary ammonia and urea.

End products and [1-^{13}C]leucine.

(calculation 5) Within the cell, [1-^{13}C]leucine is reversibly deaminated to its keto-analogue, [^{13}C]α-KICA. Through decarboxylation of [^{13}C]α-KICA to isovaleryl-CoA, $^{13}CO_2$ is liberated as a respiratory "end-product" of leucine oxidation. The latter step is irreversible.

When using the recovery of label in expiratory air during administration of [1-^{13}C]leucine for calculating whole-body protein turnover, one assumes that the ratio of oxidation of leucine (E_L) to total body protein oxidation (E_N) is equal to the ratio of dietary intake of leucine (I_L) to dietary intake of protein (I_N) [18,19]. In equation:

$$\frac{E_L}{I_L} = \frac{E_N}{I_N} \quad (5)$$

Total body protein oxidation is measured by the nitrogen excretion (E_N).
Since intake of leucine and total amount of nitrogen in the diet can be calculated from the composition of the formula and the nitrogen excretion can be measured in the urine, the total amount of leucine oxidized can be calculated.
Leucine oxidation also equals the leucine flux times the recovery of label in expiratory air.

The equation used for the oxidation is:

$$leucine\ oxidation = \left(\frac{VCO_2 \times IE_{CO_2}}{I \times c}\right) \times Q \qquad (6)$$

where VCO_2 is the rate of CO_2 excretion in μmol· kg^{-1}· min^{-1} during the [1-^{13}C]leucine infusion, IE_{CO_2} is the isotopic enrichment of expired CO_2 at plateau, I is the [1-^{13}C]leucine infusion rate in μmol [^{13}C]· kg^{-1}· min^{-1} and c is the correction factor for bicarbonate retention calculated from the energy intake [27]. If the leucine oxidation is derived from equation 5 and recovery of the label in expiratory air (the first part of equation 6) is measured, an estimate of leucine turnover can be calculated as leucine oxidation divided by recovery of the label. Whole-body protein turnover can be calculated assuming that 590 μmol of leucine represents 1 gram of protein [28].

Analogous to the measurement of [^{15}N] enrichment in urea or ammonia, the [^{13}C] enrichment in expiratory air requires an IRMS as well.

Direct measurement and [1-^{13}C]leucine

(calculation 6) With the infusion of [1-^{13}C]leucine and the measurement of labelled α-KICA, one applies the reciprocal pool model to calculate whole-body protein turnover [29,30]. The dilution of the tracer in plasma is an estimate of the turnover of the amino acid, in equation:

$$Q = i \times \left(\frac{E_i}{E_p} - 1\right)\ \mu mol \cdot kg^{-1} \cdot h^{-1} \qquad (7)$$

where i is the [1-^{13}C]leucine infusion rate (μmol.kg^{-1}.hr^{-1}) and E_i is the isotopic enrichment of the [1-^{13}C]leucine infused (atom%excess) and E_p is the isotopic enrichment of α-KICA in plasma. Leucine flux is extrapolated to whole-body protein turnover using again the assumption that 1 gram of protein contains 590 μmol leucine. The [^{13}C] enrichment of α-KICA is measured by a gas chromatograph mass spectrometer (GCMS).

To calculate whole-body protein synthesis and breakdown from whole-body protein turnover the equation used is:

$$Q = S + E = I + B \qquad (8)$$

Assuming a constant pool size, nitrogen leaving the pool (for protein synthesis (S) and urinary excretion (E)) should equal nitrogen entering the pool (from dietary intake (I) and from protein breakdown (B)). With the measurement of total nitrogen excretion and nitrogen intake, both synthesis and breakdown rates can be obtained if the flux (Q) is known. Values for whole body protein are calculated by multiplying the nitrogen data by a factor of 6.25.

(calculation 7) If one does not want to extrapolate to whole-body protein rates, other terms are used, that are directly derived from data obtained by leucine turnover studies. Non-oxidative disposal (NOD, a measure of protein synthesis) and leucine released from proteins (LRP, a measure of protein breakdown) can be calculated according to the following equation:

$$Q_{leu} = Ox_{leu} + NOD_{leu} = I_{leu} + LRP \qquad (9)$$

Again, these leucine turnover rates can be extrapolated to whole-body protein turnover rates [28]. It is not needed to measure nitrogen excretion using this calculation. Both an IRMS and a GCMS are needed to measure the [^{13}C] enrichment in expiratory air and the [^{13}C] enrichment in α-KICA respectively.

STATISTICS

Data are presented as mean $\pm$ SD. The differences between the estimates of whole-body protein turnover rates with the six different methods were analyzed by one-way analysis of variance with the Bonferroni correction for multiple tests. Correlations between measurements were tested with Spearman rank correlation tests. Statistical significance was set at a level of $P < 0.05$.

RESULTS

The AGA and SGA infants differed in birth weight, gestational age and weight at study (**Table 3.1**). Since we did not find a significant effect of energy intake on whole-body protein turnover, we did not subdivide the AGA and SGA infants in different energy intake groups. As shown, both energy intake and protein intake were not different between AGA and SGA infants.

Table 3.2. Rates of whole-body protein flux, protein synthesis and protein breakdown estimated from different tracers and calculations. All rates are given in g protein per kilogram per day.

1: administration of [^{15}N]glycine and measurement of ^{15}N-urea enrichment,

2: administration of [^{15}N]glycine and measurement of ^{15}N-ammonia enrichment,

3: administration of [^{15}N]glycine and taking an arithmatic mean of 1 and 2,

4: administration of [^{15}N]glycine and taking an harmonic mean of 1 and 2,

5: administration of [1-^{13}C]leucine and measuring the enrichment in expiratory air,

6: administration of [1-^{13}C]leucine and measuring the enrichment in plasma. Synthesis and breakdown rates are obtained from the nitrogen excretion and protein intake,

7: administration of [1-^{13}C]leucine and measuring the enrichment in plasma and in expiratory air. Synthesis and breakdown rates are obtained from equation 9.

	AGA			SGA		
	Q	S	B	Q	S	B
End-products						
1 urea	12.6±2.0	11.7±2.2	9.7±2.0	-	-	-
2 NH_3§	10.4±1.1	9.5±1.2	7.5±1.1	12.9±2.6‡	12.3±2.6‡	10.0±2.6‡
3 AA	11.5±1.3	10.6±1.5	8.6±1.3	-	-	-
4 HA	11.3±1.2	10.4±1.4	8.4±1.2	-	-	-
5 CO_2†	6.0±2.2	5.1±2.0	3.1±2.2	4.7±1.2	4.1±1.1	1.8±1.1
Direct						
6 KICA§	14.0±3.0	13.1±3.1	11.1±3.0	13.4±5.2	12.8±5.1	10.5±5.0
7 KICA + CO_2§	14.0±3.0	11.2±2.4	9.9±3.0	13.4±5.2	11.2±4.5	9.3±5.0

‡ indicates a significant difference between AGA and SGA infants at $P < 0.05$,

† indicates a significant difference between calclation 5 and the others at $P < 0.0001$, § indicates a difference at $P < 0.05$ between both calculations.

Glycine and leucine intakes, calculated from the amino acid composition provided by the manufacturer, were 32 ± 1 μmol.kg^{-1}.hr^{-1} (58 ± 2 mg.kg^{-1}.d^{-1}) and107 ± 4 μmol.kg^{-1}.hr^{-1} (339 ± 13 mg.kg^{-1}.d^{-1}), respectively. All infants were growing at the time of the study, with no statistically significant differences between the groups (**Table 3.1**).

We found plateaus in the enrichment of expiratory CO_2 after 1-2 hours of [1-^{13}C]leucine infusion (**Fig 3.1**). Enrichment of urinary ammonia usually reached plateau between 12 - 30 hours, whereas urinary urea enrichment showed a plateau between 48-72 hours (**Fig 3.2**). No significant enrichment and/or plateau of urea was found in the majority of the SGA infants (seven out of nine). Those infants had, compared to the infants who showed a plateau in urinary urea enrichment, a lower study weight (1.3±0.1 *vs*. 1.5±0.2 kg, $P = 0.01$) and higher gestational age (32±2 *vs*. 30±1 wk, $P = 0.005$), but they were also younger at the time of the study (22±4 *vs* 30±8 days, $P = 0.03$) and had a lower leucine oxidation (48±16 *vs* 71±22 μmol.kg^{-1}.hr^{-1}, $P = 0.025$).

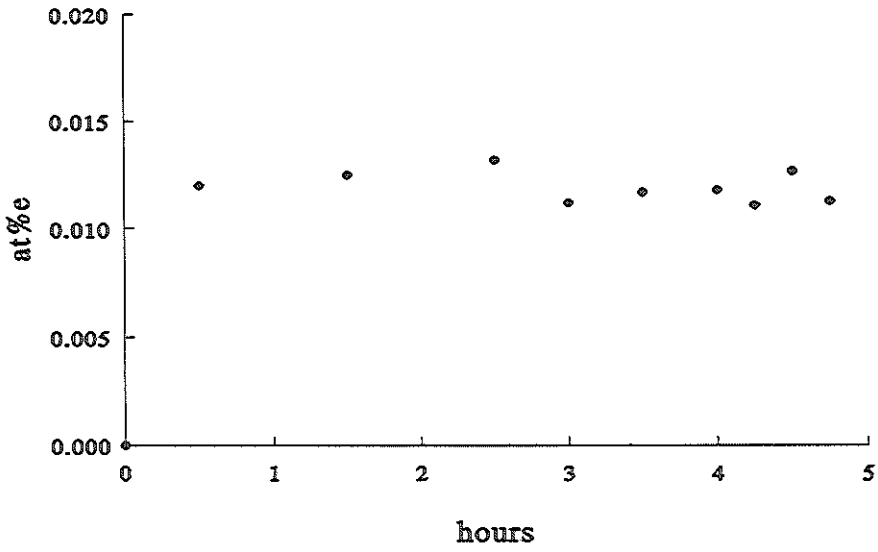
Whole-body protein turnover rates ranged from 10 to 14 g.kg^{-1}.d^{-1}, except for the rate calculated from the urinary nitrogen excretion and the enrichment of expiratory CO_2 (calculation 5), which was much lower than all the other estimations ($P < 0.0001$, **Table 3.2**). Whole-body protein turnover based upon enrichment in ^{15}N-ammonia was lower than whole-body protein turnover calculated using enrichment in plasma α-KICA, **Table 3.2** ($P = 0.024$), both being however in the range of 10 - 14 g.kg^{-1}.d^{-1}.

Table 3.3 denotes the relation between the turnover rates from the different calculations calculated for the individual patients. Only turnover rates calculated from the α-KICA-enrichment and from the harmonic average of turnovers based upon enrichment of urinary ammonia and urea showed a weak positive correlation.

Leucine kinetics are shown in **Table 3.4**. We found no correlation between the nitrogen excretion and leucine oxidation ($r = 0.12$).

Differences in whole-body protein turnover between AGA and SGA infants were only found using calculation 2 (enrichment NH_3), with a higher turnover in SGA infants. SGA infants had lower nitrogen excretion rates than AGA infants (94 ± 18 mg.kg^{-1}.d^{-1} SGA *vs* 142 ± 42 mg.kg^{-1}.d^{-1} AGA, $P = 0.001$), but the percentage of protein retention per gram new tissue was not statistically different: 13 ± 3% for AGA infants and 13 ± 1% for SGA infants.

Figure 3.1. An example of enrichment in expiratory air following a [1-^{13}C]leucine infusion.



DISCUSSION

The advantage of methods based upon end-products is the non-invasive character of the studies. This is especially important for studies in preterm infants with a limit to the amount of blood that can be drawn for study purposes. Except for the difference between end-product measurement *versus* direct measurement, it is also possible to make a distinction between the methods relating to the tracer used ([^{15}N]glycine *vs.* [1-^{13}C]leucine). The calculation of whole-body protein turnover from the turnover of the total amino acid pool as is done using [^{15}N]glycine (calculations 1-4) or from the kinetics of a specific amino acid (calculations 5,6) is fundamentally different. The [^{15}N] product method assumes labelling of the total amino acid pool by means of exchange of nitrogen between the metabolically active amino acids. The assumptions in case of calculations with the use of [1-^{13}C]leucine are different. Because leucine is an essential amino acid, it is assumed that the rate of appearance of leucine (α-KICA) is a reflection of release of all amino acids from body proteins, since there is no de novo synthesis of leucine. Both methods have their limitations.

Glycine. The [^{15}N]glycine method assumes that the total active body pool of amino acids consists of one or two metabolically active pools from which urea and ammonia are derived. The label [^{15}N] enters the pool and is assumed to be rapidly exchanged between all metabolically active amino acids. Urea and ammonia are assumed to be arising from this pool. Urea is synthesized in the liver via the urea cycle [31]. Nitrogen enters this cycle by carbamoylphosphate and aspartate. Nitrogen for aspartate arises from the α amino group of glutamate and the nitrogen for carbamoylphosphate is derived from ammonia. The latter could be derived from the amide groups of glutamine and asparagine or by degradation reactions of serine threonine, histidine, glycine and methionine. Ammonia from the bacterial decomposition of urea in the gut can also be a significant precursor. Glutamate receives its nitrogen from the majority of amino acids, but not from glycine and serine, during the following rapid conversion that is mediated by glutamate transaminase:

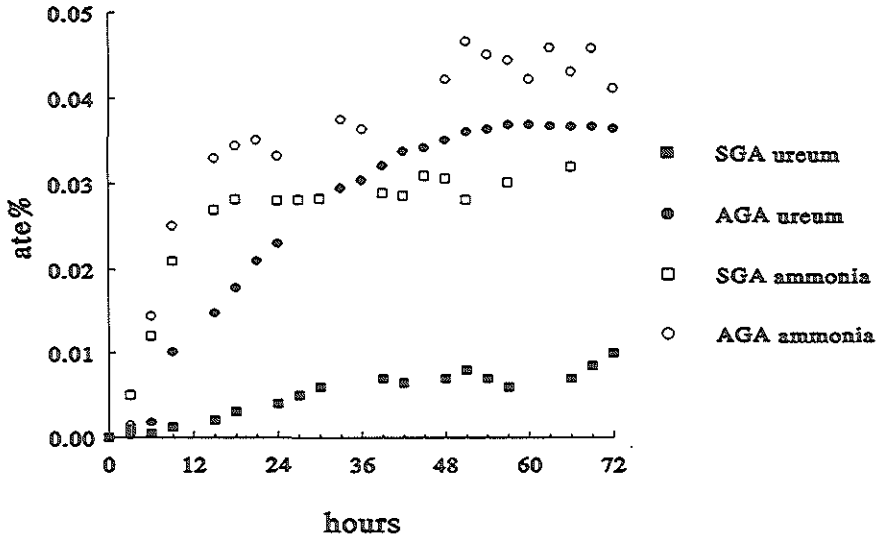
$$\alpha\text{-}amino\,acid + \alpha\text{-}ketogluterate \rightleftarrows \alpha\text{-}keto\,acid + glutamate$$

Nitrogen in the urea molecule could therefore be derived from many different amino acids.

Glycine and serine are readily transformed into one another. In addition glycine may arise from threonine by a specific aldolase enzyme or from choline. However, the two last pathways are not likely to be quantitatively important [32]. Glycine does not transaminate with glutamate as shown by Jackson and Golden who observed no enrichment of glutamate during continuous infusion of [^{15}N]glycine [33]. However, Matthews *et al.* showed labelled nitrogen in glutamate following [^{15}N]glycine administration [34], but a majority of the amino acids was not significantly enriched. Nitrogen (labelled) from glycine will probably not exchange to a great extent with a large group of amino acids via transamination which is considered to be a major pathway for rapid nitrogen transfer. For these reasons, doubt can be raised whether glycine is a suitable carrier to introduce labelled nitrogen in the metabolic nitrogen pool.

Glycine is deaminated via the glycine cleavage system, with the release of free ammonia [35]. Duda and Handler showed that free ammonia is considerably more rapidly incorporated into the amide position of glutamine than into any other nitrogenous component of liver [36]. After administration of amide N labelled glutamine, isotope appeared only slowly in urea or glutamic acid. Other studies have shown that urinary ammonia is mainly produced from the amide N of glutamine in

Figure 3.2. Examples of [^{15}N] enrichment in urinary ammonia (open symbols) and urea (filled symbols) for AGA (○) and SGA (□) infants.



the kidney [37-39].

A rapid transfer of glycine nitrogen to urinary ammonia via glutamine is therefore to be expected as was observed in both SGA and AGA infants in the present study. The question remains why we did not find enrichment of urinary urea in SGA infants.

SGA infants had lower urinary nitrogen excretion rates, of which urea is the major component, suggesting that the urea synthesis rate was lower in this group. Regulation of the urea production might be a nitrogen conservation mechanism, occurring to a greater extent in SGA infants than in AGA infants. This hypothesis is supported by the indices of protein turnover. The efficiency of protein gain (leucine retention divided by leucine turnover) was significantly higher in infants who showed low urea enrichments (21±11% (low urea enrichment) *vs* 10±8% (plateau in urea enrichment), $P = 0.03$). This is in accordance with the original work of Picou and Taylor-Roberts, who also showed a higher S/Q ratio in malnourished patients [2], and also in accordance with Cauderay *et al.* who showed a more efficient protein gain/protein synthesis ratio in SGA infants [40]. In this context, SGA infants can also be considered as malnourished infants. Cauderay *et al.* used [^{15}N]glycine, but

reported significant urinary urea enrichments in all SGA infants in contrast to the findings in the present study. However, they administered very large amounts of [^{15}N]glycine, 30 mg.kg^{-1}.d^{-1}, which is no longer a tracer dose and may have resulted in an overload of glycine. In a previous study of preterm infants who were not receiving amino acids during the first few days after birth, we also observed very low enrichment of urinary urea after [^{15}N]glycine administration [10]. Also in these infants the production of urea could have been lower. Infants who received amino acids, did have significant enrichment in urinary urea.

Glycine is a major component of haem and is also important for purine synthesis, whereas carbamoyl phosphate and aspartate, both urea cycle intermediates, are used for the synthesis of pyrimidines. With an increased need for those components, a large part of label could follow that pathway with a resulting low enrichment of urea. Purines are degraded to uric acid and with the degradation of pyrimidines, the amino group will be removed by transamination.

In conclusion we can assume that ammonia and a significant part of urea nitrogen arise from very different pools of amino acids and it is therefore not

Table 3.3. The relative percentage of protein turnover (x/y) is depicted in the left lower half of the table, the correlations between protein-turnover in individual patients (Spearman) are shown in the upper right half of the table. Significance at $P < 0.05$ is indicated by ‡.

	Q_{urea}	$Q_{ammonia}$	Q_{aa}	Q_{ha}	Q_{CO2}	Q_{kic}
	Spearman rank correlation coefficient					
Q_{urea}		0.35	0.84	0.78	-0.04	0.22
$Q_{ammonia}$	123 ± 6		0.76	0.83	-0.52‡	0.05
Q_{aa}	110 ± 2	90 ± 2		0.98	-0.15	0.47
Q_{ha}	112 ± 3	92 ± 2	102 ± 1		-0.26	0.49‡
Q_{CO2}	239 ± 23	234 ± 21	218 ± 20	215 ± 20		0.14
Q_{kic}	94 ± 6	89 ± 6	85 ± 5	84 ± 5	43 ± 4	
relative protein turnover in percentages (x ± SEM)						

surprising that differences are obtained using both methods as has been suggested by Waterlow *et al.* [41]. Averaging the results obtained via ammonia and urea enrichment, as is done in calculation 3 and 4, is likely to be not correct as well for the same reasons as discussed above. The original concept of rapid exchange of the nitrogen of glycine with other amino acids does not appear to be valid, suggesting that protein turnover as assessed by [^{15}N]glycine is unlikely to represent accurately whole-body protein turnover.

Leucine. The original model for quantification of leucine kinetics assumes a single pool from which leucine is used for protein synthesis and into which it enters from protein breakdown. Leucine turnover is calculated from the isotope dilution of the labelled leucine in plasma. The fundamental problem with this model is that the leucine tracer is infused into and sampled from plasma, while it is metabolized within the cells. Because leucine is transaminated reversibly within the cell to α-KICA, the enrichment of α-KICA reflects the intracellular enrichment of leucine [42]. Recent animal studies have shown a very good agreement of plasma α-KICA enrichment and intracellular leucine enrichment [43,44]. Intracellular enrichment is a better reflection of the precursor pool for synthesis. Recent studies have shown that a multi-pool model gives the best fit for the intracellular turnover. The multi-pool model demands however many sample points and is therefore hardly feasible in premature infants. In specific circumstances the reciprocal pool model has found to be in good agreement with a 7 pool model that can be constructed from simultaneous infusion of labelled leucine and α-KICA [45,46]. It is unclear whether data obtained for leucine kinetics can be extrapolated to whole-body protein turnover, synthesis and breakdown, because leucine is predominantly metabolized in the muscle, and therefore may not be the best representative of protein metabolism in other organs.

Comparison of calculations. From the above it is clear that a comparison of methods, as to decide which of the methods is "best" may not be meaningful, since calculations based on enrichment of urea, ammonia and α-KICA are reflections of different processes.

The end product method of estimation of whole-body protein turnover from urinary nitrogen excretion and from expiratory CO_2 after [1-^{13}C]leucine administration (calculation 5) gave results that were significantly lower as compared with all the other calculations. This calculation assumes a correlation between leucine oxidation and nitrogen excretion in urine. We measured the leucine oxidation quantitatively and found that the leucine oxidation did not correlate with the urinary nitrogen excretion. Hence, this calculation is not suitable for the estimation of

Table 3.4. Leucine kinetics in AGA and SGA infants. Non-oxidative disposal represents protein synthesis, leucine release from protein is analogous to protein breakdown.

	AGA	SGA
Turnover (μmol.kg^{-1}.hr^{-1})	345 ± 74	329 ± 127
Recovery of [^{13}C] in expired air (%)	20 ± 4	17 ± 2
Oxidation (μmol.kg^{-1}.hr^{-1})	70 ± 22	54 ± 18
Non-oxidative disposal (μmol.kg^{-1}.hr^{-1})	275 ± 60	275 ± 110
Leucine release from protein (μmol.kg^{-1}.hr^{-1})	243 ± 74	228 ± 123

whole-body protein turnover in this group of patients, although it has been used in preterms [19] and elderly [18]. The total amount of leucine oxidation could be influenced by the diet, since the leucine concentration of the protein in the diet exceeded the leucine tissue concentration in our study. This could have led to an increased leucine oxidation, but not to a difference in oxidation between AGA and SGA infants since both groups received the same amount of protein.

Although most group means were not significantly different, the turnover rates obtained by measurement of plasma α-KICA enrichment (calculation 5) were higher than the other rates. The duration of tracer administration is much shorter as compared to the duration of [^{15}N]glycine administration. Recycling of the tracer in the latter experiments could increase the enrichment, with a corresponding lower turnover rate. Estimation of recycling of [^{15}N]glycine by Cauderay *et al.* was 20% after 72 hours of infusion [40], whereas Schwenk *et al.* calculate 30% recycling of a labelled essential amino acid after 24 hours of infusion [47].

The individual turnover rates, obtained from the different calculations, did not correspond well. As is shown in table 3.3, the only significant correlation between non-related estimates of whole-body protein turnover was found between the harmonic average (calculation 4) and the α-KICA enrichment calculation (calculation 6).

Route of tracer administration. In this study we administered the tracer by the same route as the feed. Some considerations have to be made when using intragastric

infusion of label. The intestinal absorption must be known. The [^{15}N]glycine excretion in faeces has been measured before and is 0.3 - 3% of the intake [2,8]. Another possible confounding factor is the uptake in the splanchnic area. With a considerable uptake of tracer and tracee in the splanchnic area, the amount of tracer that reaches the circulation is reduced. Subsequently, this amount is more easily diluted by the systemic flux, resulting in a lower enrichment. Both protein turnover and proteolysis could therefore be overestimated. Hoerr *et al.* performed a study with simultaneous administration of labelled leucine intravenously and intragastricly in adults [48]. With the measurement of α-KICA in plasma they found no significant differences in enrichment between iv and ig administration. Although almost 20% of the leucine was taken up by the splanchnic area, it appeared that the leucine was transaminated and released as α-KICA. Beaufrère *et al.* performed a similar experiment in preterm infants but found higher splanchnic extraction rates of 35-48%, depending on measurement of ^{13}C-KICA or [1-^{13}C]leucine, respectively [49]. Picou, Taylor-Roberts performed iv and ig tracer studies with [^{15}N]glycine on separate days in two infants [2]. The calculated values for whole-body protein turnover were slightly higher (3% and 14%) when [^{15}N]glycine was administered orally. The whole-body protein turnover values in the present study could thus well be an overestimation.

Conclusions

1) Because whole-body protein consists of heterogenous pools of amino acids with different rates of turnover and possibly different amino acid composition, no single amino acid will represent whole-body protein turnover.
2) In SGA infants, urinary ammonia becomes enriched after continuous administration of [^{15}N]glycine. Urinary urea shows hardly any enrichment, which might be due to a decreased rate of urea synthesis. This suggests a protein sparing mechanism in SGA infants, which is supported by a decreased nitrogen excretion and a decreased leucine oxidation found in this group of infants.
3) Group averages for whole-body protein turnover in orally fed preterm infants ranged between 10 to 14 g.kg^{-1}.d^{-1}, except for the calculation based upon a correlation between leucine oxidation and nitrogen excretion.
4) Leucine oxidation is not correlated with nitrogen excretion.

5) We found no correlation between whole-body protein turnover measurements with [^{15}N]glycine or [1-^{13}C]leucine within individual patients.

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CHAPTER 4

IMMEDIATE COMMENCEMENT OF AMINO ACID SUPPLEMENTATION IN PRETERM INFANTS: EFFECT ON AMINO ACID LEVELS AND PROTEIN KINETICS ON THE FIRST DAY OF LIFE.[33]

ABSTRACT

There is a general reluctancy to start amino acid administration in preterm infants from birth onwards. This might lead to loss of lean body mass and impairment of growth. We measured amino acid levels and protein kinetics in 18 preterm infants on postnatal day 1. Nine infants (group "G+AA") received amino acids (1.15 $\pm$ 0.06 $g.kg^{-1}.d^{-1}$) and glucose (6.05 $\pm$ 1.58 $g.kg^{-1}.d^{-1}$), whereas the other nine infants (group "G") received exclusively glucose (6.48 $\pm$ 1.30 $g.kg^{-1}.d^{-1}$) within a few hours of birth. Protein kinetics were measured using a stable isotope dilution technique with [1-^{13}C]leucine as a tracer.

No statistically significant differences were noticed in pH, base excess, blood urea concentration and glucose levels. Total amino acid concentration as well as total essential amino acid concentration were significantly different and were below the reference range in the non-supplemented group. Plasma amino acid levels of five essential amino acids were below the reference range in the non-supplemented group (methionine, cystine, isoleucine, leucine, arginine), whereas only cystine was below the reference range in the supplemented group.

Nitrogen retention improved significantly by the administration of amino acids (-110 $\pm$ 44 mg $N.kg^{-1}.d^{-1}$ "G" *vs.* +10 $\pm$ 127 $mgN.kg^{-1}.d^{-1}$ "G+AA", P = 0.001), while the leucine oxidation was not significantly increased in the supplemented group (41 $\pm$ 13 $\mu mol.kg^{-1}.hr^{-1}$ "G" *vs.* 46 $\pm$ 16 $\mu mol.kg^{-1}.hr^{-1}$ "G+AA"). The leucine balance improved significantly (-41 $\pm$ 13 $\mu mol.kg^{-1}.h^{-1}$ "G" *vs.* -8 $\pm$ 16 $\mu mol.kg^{-1}.hr^{-1}$ "G+AA", P = 0.01) due to a combination of increased amount of leucine being used for protein synthesis and a lower amount of leucine coming from protein breakdown. Plasma cystine concentration, the only amino acid below the reference range in the supplemented group, was highly predictive for protein synthesis in that group.

We conclude that the administration of amino acids to preterm infants from birth onwards seems safe and prevents the loss of protein mass.

INTRODUCTION

The fetus in utero continuously receives amino acids, which are used for protein synthesis [1]. Following birth, this flow is interrupted and amino acid administration is usually not started before the third day of life in the preterm infant [2]. This leads to a loss of lean body mass, while the stress of birth, infections and handling

procedures might add to the protein catabolism. The reluctance to start amino acid administration soon after birth is due to the increased risk of hyperammonemia, metabolic acidosis and uremia. These side effects, caused by the immaturity of metabolic pathways of preterm infants, have been reported in association with the use of protein hydrolysates [3,4]. Although protein hydrolysates have been replaced by crystalline solutions, still reluctancy exists to the introduction of these solutions during the first days of life. Early introduction of amino acids could have a beneficial effect on nitrogen balance and recent studies show no adverse effects [5,6].

Nitrogen balances are useful to determine the overall effect of supplementation, but no information has been obtained on protein synthesis or breakdown. Specific amino acids labelled with stable isotopes, especially [^{15}N]glycine and [1-^{13}C]leucine, have been used to gain insight in the kinetics of protein metabolism. Preterm infants showed an improved protein balance by an increase in protein synthesis, measured several days after the start of the amino acid supplementation with energy intakes of 30-50 kcal.kg^{-1}.d^{-1} [7,8]. Nonprotein energy should however be supplied in sufficient amounts to utilize the supplemented protein [9], but the energy intake during the first day of life is usually limited. The effect of amino acid supplementation on protein synthesis could thus be reduced in such conditions. Furthermore, scanty data is available on whether preterm infants can tolerate the amino acid load from birth onwards.

The present study was designed to gain information on protein kinetics following amino acid supplementation at very low energy intakes on the first day of life. Leucine kinetics, nitrogen balances and amino acid profiles were determined in preterm infants.

PATIENTS

Eighteen preterm infants were studied. The clinical data are shown in **Table 4.1.** Gestational age was calculated from maternal history or, in case of uncertainty, estimated from the Ballard score [10]. Patients were defined appropriate for gestational age if the weight was between -2 SD and + 2 SD according to the charts of Usher and McClean [11]. Infants with birth weights below 2 SD were considered to be small for gestational age. All infants needed mechanical ventilation at the start of the study and had indwelling arterial and venous catheters. None of the infants had congenital abnormalities.

Table 4.1. The clinical characteristics of the patients studied. Mean ± 1SD, or in cases of apparent non-normality as median [range].

	glucose (n=9)	glucose + amino acids (n=9)
birth weight (g)	1439 ± 274	1356 ± 338
gestational age (wk)	29.4 ± 1.7	29.1 ± 1.5
apgar score (5 min)	7.8 ± 1.4	8.6 ± 1.7
female:male	2:7	2:7
study age (hrs)	21.5 ± 3.1	19.8 ± 4.3
ventilatory score	60 [6 - 300]	22 [0 - 738]
pH	7.36 ± 0.06	7.34 ± 0.04
base excess	-3.6 ± 1.7	-4.0 ± 1.0
ml (4.2% w/v) $NaHCO_3$/d administered	3 [0 - 15]	4 [0 - 13]
glucose (mmol/L)	5.0 ± 1.5	4.2 ± 1.1
urea (mmol/L)	5.0 ± 2.1	6.2 ± 3.2

The infants were scored according to an adapted ventilatory scoring system, as described previously [12]:

$$VS = VF \times MAP \times FiO_2$$

where VS is the ventilatory score, VF the ventilatory frequency, MAP the mean airway pressure and FiO_2 the oxygen ratio in inspiratory air.

Nine infants received glucose (10% anhydrous glucose (w/v)) and amino acids (Primène 10%, Clintec, Baxter, Benelux NV, Brussels, Belgium) within a few hours after birth, group "G + AA", whereas nine infants received exclusively glucose, group "G". Nutrient and energy intake are shown in **Table 4.2.**

Informed consent was obtained from at least one of the parents prior to the start of the study. The use of stable isotopes for human investigations had been approved by the ethical committee of the Academic Hospital/Erasmus University

Rotterdam.

All patients were treated with prophylactic antibiotics although no positive blood cultures were found afterwards. Corticosteroids (betamethasone) had been given antenatally to four mothers to accelerate the lung maturation in group "G + AA", and to five mothers in group "G".

METHODS

Isotope infusion. [1-^{13}C]leucine (99% enriched, Isotech, Miamisburg, Ohio, USA) and $NaH^{13}CO_3$ (98.7% enriched, Isotech, Miamisburg, Ohio, USA) were diluted in 0.9% NaCl by the hospital's pharmacy. Both solutions were filtered through a 0.22μ Millipore filter and put into 5 ml sterile vials. [1-^{13}C]leucine concentration in the infusate was measured by an amino acid analyzer. $NaH^{13}CO_3$ concentration in the infusate was checked by isotope ratio mass spectrometry (SIRA 10, Fisons, VG Isotech Lt. Middlewich, Chesire, UK).

A priming dose of 6.9 μmol $NaH^{13}CO_3$/kg was followed by a continuous infusion of 4.7 μmol/kg.hr. This infusion lasted for two hours and was followed by a [1-^{13}C]leucine priming of 2 mg/kg. Continuous infusion of [1-^{13}C]leucine (2 $mg.kg^{-1}.hr^{-1}$) thereafter, lasted for 3 hours. Tracers were given using a Harvard infusion pump (M22, Harvard Apparatus Co Inc S. Natick, MA, USA) and administered via the same infusion route as the nutrients.

Arterial blood samples were drawn before the infusion of the stable isotopes, at 30 minutes before the end and at the end of the [1-^{13}C]leucine infusion. The samples were immediately put on melting ice and centrifugated at 4°C. Plasma was divided into aliquots and stored at -70°C. The whole procedure did not exceed 15 min.

Measurement of isotope dilution in plasma. Leucine is reversibly transaminated to its keto-analogue: α-keto-isocaproic-acid (α-KICA) within the cell [13,14]. The plasma enrichment of α-KICA is very close to intracellular [1-^{13}C]leucine enrichment [15,16]. Measurement of the enrichment of α-KICA following [1-^{13}C]leucine infusion will therefore reflect the site of incorporation of leucine in proteins as well as the site for decarboxylation of α-KICA to isovaleryl-CoA.

One hundred μL of plasma was deproteinized by adding 250 μl sulfosalicylic acid (6% (w/v)), after the addition of the internal standards ketocaproic acid and ketovaleric acid. After keeping the samples for 5 min at 0°C, the samples were centrifugated and 150 μL 1,2-phenylenediamine (2 g%) dissolved in 6 M HCL was

added to the supernatant. The tubes were heated at 100°C for 60 minutes and afterwards allowed to cool. After addition of 3 ml dichloromethane-hexane mixture (1:1, v/v) and mixing, the supernatant was evaporated to dryness under nitrogen at 50°C. Derivatisation of the keto amino acids was performed by adding 50 μl *N,O-bis*(trimethylsilyl)trifluoroacetamide after which the vials were immediately sealed under nitrogen and left at room temperature for 30 minutes.

Gas chromatography/mass spectrometry was carried out on a Hewlett Packard HP 5890 (Palo Alto, CA, USA) gas chromatograph coupled to a JEOL JMS-DX303 (Tokyo, Japan) mass spectrometer operating in the electron impact mode. Chromatography was carried out on a 25 m x 0.32 mm capillary column (CP-Sil5-CB, Chrompack BV, Middelburg, The Netherlands). Selective ion monitoring was carried out at m/z 232/233 for (m) and (m+1) respectively. The plasma α-KICA enrichments were determined using a calibration graph constructed from standard mixtures ranging from 0 to 20 mol% ^{13}C-α-KICA. The coefficient of variation for plasma α-KICA measurements was 2% (n=5). α-KICA enrichments were calculated as mol percent excess above baseline enrichment.

Leucine oxidation measurement. Expiratory CO_2 was collected at the outlet of the ventilator. Air leaving the ventilator was lead through an all glass spiral filled with freshly prepared NaOH (1.0 N). Six baseline expiratory air samples were taken to determine the natural abundance of $^{13}CO_2$. One hour after the primed $NaH^{13}CO_3$ infusion started, 5 minute CO_2 collections were performed at 10 minute intervals for one hour. After the [1-^{13}C]leucine infusion was started, CO_2 collections were made at hourly intervals for the first two hours and at 10 min intervals for the final hour. Breath samples were analyzed for enrichment of $^{13}CO_2$ on a isotope ratio mass spectrometer (VG SIRA 10, Fisons Instruments, Middlewich, Cheshire, UK) as described earlier [12].

Plasma analyses. Amino acid levels were determined on a LKB 4151 Alpha plus Amino Acid Analyser (Pharmacia LKB Biochrom Ltd., Cambridge UK) in plasma taken at the start of the study. The plasma was deproteinized with sulfosalicylic acid. Plasma glucose concentration was analyzed on a Cobas Mira (Roche, Basel, Switzerland).

Urinary nitrogen excretion. The usage of adhesive bags in very young premature infants is detrimental to the skin and we collected therefore the diapers (Pampers, Procter and Gamble, Vlaardingen, The Netherlands) for 12 to 24 h, as previously described [17]. Briefly, the diapers were soaked with a citrate buffer and a fixed sample from each soaked diaper was taken. The samples were mixed and freeze-

dried. The total nitrogen content was measured on an automatic nitrogen analyser (ANA 1400, Carlo Erba, Milan, Italy). Nitrogen excretion in the group receiving amino acids was corrected for changes in body urea nitrogen as is described by Mitton *et al.* [18].

Calculations. The leucine flux was calculated from the dilution of the ^{13}C-α-KICA according to the following equation:

$$Q_L = I \times \left(\frac{E_i}{E_p} - 1 \right)$$

where Q_L is the total turnover rate of leucine in μmol.kg^{-1}.hr^{-1}, I is the leucine infusion rate in μmol.kg^{-1}.hr^{-1}, E_i is the enrichment of [1-^{13}C]leucine in the infusate in mol% excess and E_p is the plasma enrichment of ^{13}C-α-KICA at plateau in mol% excess. In steady state the amount of leucine entering the plasma pool should be equal to the amount of leucine leaving the pool. Leucine can enter the pool when released from proteins as a result of protein breakdown or through the diet. Leucine leaving the pool may be either oxidative disposal or non-oxidative disposal (leucine used for protein synthesis). In equation:

$$Q_L = I + LRP = O + NOD$$

where I is the total leucine intake in μmol.kg^{-1}.hr^{-1}, LRP is the amount of leucine released from protein *via* protein breakdown in μmol.kg^{-1}.hr^{-1}, O is the rate of leucine oxidation in μmol.kg^{-1}.hr^{-1} and NOD is the rate of non-oxidative leucine disposal (a measure of protein synthesis rate) in μmol.kg^{-1}.hr^{-1}.

Leucine oxidation was calculated by multiplying the recovery of the label in expiratory air with the rate of appearance of leucine. The recovery of the label in expiratory air was measured according to the following equation, assuming a constant VCO_2 during the study [19]:

$$Rec = \frac{IE_L \times I_B}{IE_B \times I_L}$$

where IE_L is the $^{13}CO_2$ enrichment at plateau during the [1-^{13}C]leucine infusion, IE_B is the $^{13}CO_2$ enrichment at plateau during the $NaH^{13}CO_3$ infusion, I_L is the infusion rate of [1-^{13}C]leucine in μmol ^{13}C.kg^{-1}.hr^{-1} and I_B is the infusion rate of $NaH^{13}CO_3$ in μmol^{13}C.kg^{-1}.hr^{-1}.

Table 4.2. Nutrient and energy intake.

	glucose ($mg.kg^{-1}.min^{-1}$)	amino acids ($mg.kg^{-1}.min^{-1}$)	Energy ($kcal.kg^{-1}.d^{-1}$)
Glucose (n=9)	4.5 ± 0.9	-	26.1 ± 5.5
Glucose and amino acids (n=9)	4.2 ± 1.1	0.80 ± 0.04	28.5 ± 6.2

Whole-body protein turnover (Q) was calculated from leucine turnover rate, assuming that 1 g of body protein contains 590 μmol leucine [20]. Whole body protein turnover rates are given in $g.kg^{-1}.d^{-1}$. Whole body protein synthesis (S) and breakdown (B) rates were calculated according to the following equation:

$$Q = S + E_N = B + I_N$$

where I_N is the nitrogen intake and E_N is the urinary nitrogen excretion.

Statistical analysis. Data are presented as mean ± 1 SD, or in cases of apparent non-equal distribution as median with the range in brackets. Statistical analysis was performed with non-parametric tests (Mann-Whitney U). Significance level was set at 0.05. Because of the multiple variables assessed on single samples, differences in amino acid concentrations were considered to be statistically significant at $P \leq 0.005$.

RESULTS

There were no differences in birth weight, gestational age, postnatal age and ventilatory score between the two groups (**Table 4.1**). We did not observe an effect of the amino acid administration on blood pH or base excess, nor did we find a difference in the amount of administered bicarbonate during the first 24 hours. The lowest pH in both groups was 7.28, with six infants having pH < 7.35 in group "G + AA", and four in group "G".

Both groups received on average less than 30 $kcal.kg^{-1}.d^{-1}$, with no statistically significant difference in glucose or energy intake (**Table 4.2**). The intake of the branched chain amino acids (leucine, isoleucine, valine) was 276 ± 14 $mg.kg^{-1}.d^{-1}$.

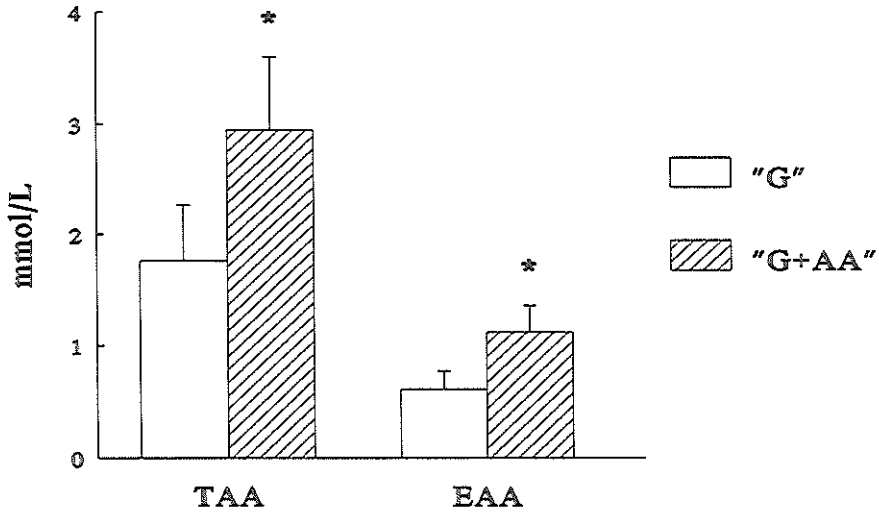
Glucose levels were 4.2 ± 1.6 mmol/L in group "G + AA" and 5.0 ± 1.5 mmol/L in group "G". Urea concentration, measured during the leucine turnover

study was not significantly different between the two groups (5.0 ± 2.1 mmol/L "group G" *vs* 6.2 ± 3.2 mmol/L "group G+AA").

The nitrogen retention is described in **Table 4.3.** Blood urea concentration increased slightly in the group infants receiving amino acids during the urine collection period. This change in blood urea concentration corresponded to an increase of 7 ± 72 mg $N.kg^{-1}.d^{-1}$ in the blood. The measured urinary nitrogen excretion was 167 ± 115 $mg.kg^{-1}.d^{-1}$. The resulting nitrogen retention (10 ± 127 $mg.kg^{-1}.d^{-1}$) was not significantly different from zero. Without amino acid administration, the nitrogen retention was negative, which was significantly different from the nitrogen retention of group "G + AA" (-110 ± 44 $mg.kg^{-1}.d^{-1}$ group "G" *vs.* +10 ± 127 $mg.kg^{-1}.d^{-1}$ group "G + AA", $P = 0.001$).

The total amino acid level increased by almost 70% ($P = 0.002$), but the percentage essential amino acids did not change statistically significant (38 ± 5% "G + AA" vs 35 ± 2 % "G", $P = 0.10$, **Figure 4.1**).

Figure 4.1. Plasma total amino acid level (TAA) and total essential amino acid level (EAA) for the supplemented and not-supplemented group. Asterix denotes a statistical significant difference at $P = 0.002$.



Without amino acid supplementation, the indispensable amino acids methionine, cystine, isoleucine and leucine were below the reference range obtained from the data from Wu et al, who reports on plasma amino acid pattern in normal term breast fed infants (**Table 4.4**) [21]. Supplementation of 1.15 g amino acids.$kg^{-1}.d^{-1}$ lead to a mean phenylalanine level above the reference range, whereas the mean cystine level was lower than the reference value. All other indispensable amino acids were within the reference range. The indispensable amino acid aspartate was slightly higher compared to the reference values in both groups, whereas arginine and glutamate were both below the reference range in group G.

The supplementation of amino acids did not result in a statistically higher leucine turnover rate (**Table 4.5**). As was described in the method section, our method relies on artificial ventilation or continuous positive airway pressure to perform oxidation studies in the infants. During the study two infants were extubated, so we were unable to measure leucine oxidation rates in these two infants. In another infant we did not obtain a plateau in enrichment of expiratory air, leaving 15 infants (eight group G, seven group G + AA) being studied for oxidation rates and subsequently nonoxidative disposal rates.

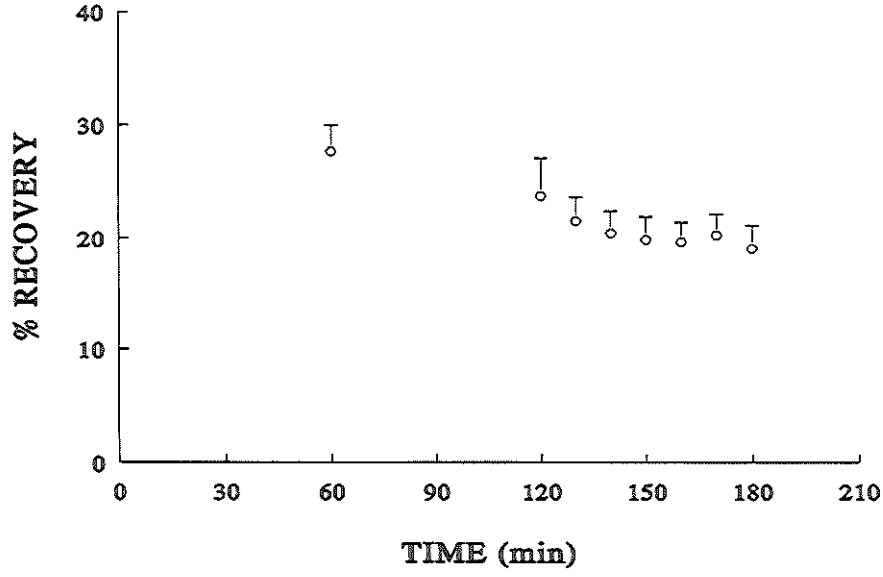
In all infants a $^{13}CO_2/^{12}CO_2$ plateau was obtained in expiratory air during both $NaH^{13}CO_3$ and [1-^{13}C]leucine infusions. The percentage of the leucine turnover that was oxidized is shown in **Figure 4.2.** We found no statistically significant difference between the two groups (21 ± 8% in group "G+AA" and 20 ± 6% in group "G"). Extrapolating the leucine oxidation rates to whole body protein oxidation rates, enabled us to calculate the contribution of protein oxidation to energy metabolism.

Table 4.3. Nitrogen balance data.

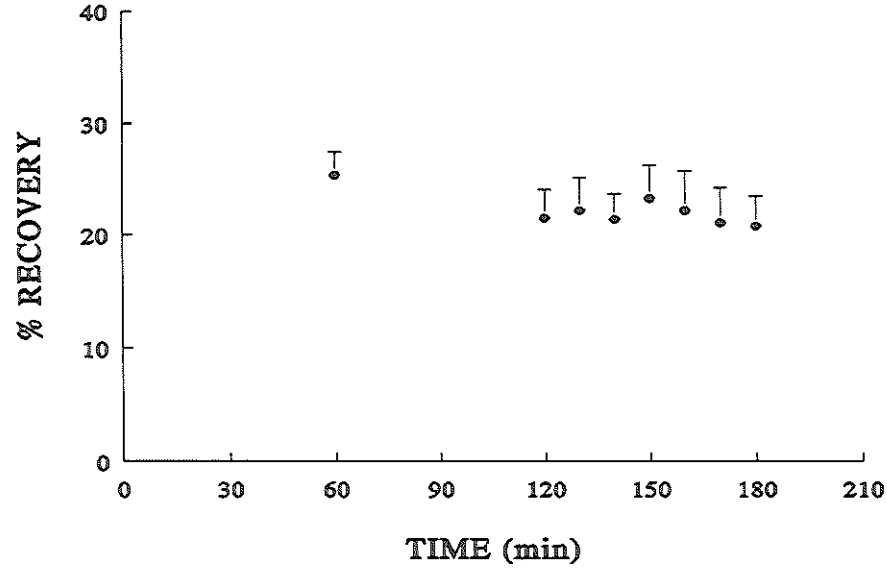
	N intake ($mg.kg^{-1}.d^{-1}$)	N excretion ($mg.kg^{-1}.d^{-1}$)	N balance ($mg.kg^{-1}.d^{-1}$)
Glucose (n=9)	-	110 ± 44	-110 ± 44
Glucose and amino acids (n=9)	184 ± 6	174 ± 123§	10 ± 127†

§ corrected for changes in blood urea concentration. †Statistically different at P=0.001.

Figure 4.2. The percentage of leucine turnover which is being oxidized throughout the [1-^{13}C]leucine infusion. a. group "G"



b. group "G+AA".



Protein oxidation provided 6.7 ± 2.1 kcal.kg^{-1}.d^{-1} in group G and 7.5 ± 2.6 kcal.kg^{-1}.d^{-1}. Leucine being released from protein via breakdown was not significantly lower in the amino acid supplemented group, nor was the amount of leucine used for protein synthesis higher in that group. The resulting leucine balance however was negative in the infants who received exclusively glucose, but not different from zero in infants who received amino acids. The difference between the two groups was statistically significant (-41 ± 13 μmol.kg^{-1}.hr^{-1} group "G" *vs.* -8 ± 16 μmol.kg^{-1}.hr^{-1} group "G+AA", $P = 0.008$).

Seventy percent of the variance in the amount of leucine used for synthesis in group "G + AA" was depending on the cystine level ($P = 0.02$, $r^2 = 0.70$, $r = 0.83$):

$$\textit{Non-oxidative leucine disposal} = 2.3\ (CYS) + 104$$

Cystine was the only, for premature infants, indispensable amino acid which was below the reference range. In group "G", multiple regression analysis only gave glutamic acid as a weak significant predictor of protein synthesis.
NOD = 201 - 2.0[glu], $P = 0.045$, $r^2 = 0.43$, $r = 0.72$. Addition of other amino acids in this regression anlysis did not result in a higher r_2, or lower P value.

Antenatally administered corticosteroids did not have a statistically significant effect on turnover rates (228 ± 42 μmol.kg^{-1}.hr^{-1} cort(+) *versus* 199 ± 27 μmol.kg^{-1}.hr^{-1} cort(-), $P = 0.10$). Leucine oxidation was also not significantly different (41 ± 8 μmol.kg^{-1}.hr^{-1} cort(+) *vs.* 46 ± 19 μmol.kg^{-1}.hr^{-1}, $P = 0.82$). The release of leucine from proteins (a measure for protein breakdown) however was statistically significant higher in the group infants whose mothers received corticosteroids (197 ± 33 μmol.kg^{-1}.hr^{-1} cort(+) *versus* 165 ± 25 μmol.kg^{-1}.hr^{-1} cort(-), $P = 0.03$). We did not find a correlation between leucine kinetics and the ventilatory score.

Whole body protein turnover was extrapolated from the leucine turnover, by assuming that 590 μmol leucine corresponds to 1 gram protein. Whole-body protein turnover tended to be higher in group "G+AA", but the difference was not significant (8.0 ± 0.9 g.kg^{-1}.d^{-1} *vs.* 9.4 ± 1.8 g.kg^{-1}.d^{-1} $P = 0.085$). Protein breakdown was not affected by amino acid supplementation (8.0 ± 0.9 g.kg^{-1}.d^{-1} group G *vs.* 8.2 ± 1.7 g.kg^{-1}.d^{-1} group G+AA). Protein synthesis was 7.5 ± 0.8 g.kg^{-1}.d^{-1} in group G and 8.3 ± 2.0 g.kg^{-1}.d^{-1} in group G+AA, $P = 0.21$.

Table 4.4. Plasma amino acid levels of the two groups with a reference range of breast-fed term infants in the fifth column [21]. Values in μmol/L or, in cases of total amounts, mmol/L. Values are depicted in bold whenever they are out of the reference range. The third column depicts the level of significance of the differences between the two groups.

	glucose	glucose + amino acids	*P* value	Term infants
Threonine	85 ± 29	165 ± 44	0.002	70 - 197
Valine	105 ± 42	201 ± 34	0.002	88 - 222
Methionine	**18 ± 7**	35 ± 11	0.005	22 - 50
Cystine	**11 ± 9**	**30 ± 11**	0.002	35 - 69
Isoleucine	**21 ± 9**	63 ± 21	0.001	27 - 90
Leucine	**45 ± 15**	104 ± 28	0.001	53 - 169
Tyrosine	100 ± 27	108 ± 40	0.39	38 - 119
Phenylalanine	60 ± 18	**87 ± 14**	0.004	22 - 72
Lysine	109 ± 33	232 ± 66	0.002	80 - 232
Histidine	66 ± 21	105 ± 45	0.03	34 - 119
Arginine	**28 ± 12**	66 ± 27	0.003	42 - 148
Aspartic acid	8 ± 2	16 ± 5	0.001	5 - 51
Serine	109 ± 38	175 ± 48	0.016	0 - 326
Aspartate	**91 ± 33**	**87 ± 29**	0.85	16 - 81
Glutamic acid	**20 ± 7**	50 ± 16	0.001	24 - 243
Glutamine	383 ± 116	546 ± 165	0.034	142 - 851
Proline	112 ± 52	189 ± 54	0.007	83 - 319
Glycine	197 ± 74	298 ± 66	0.016	77 - 376
Alanine	158 ± 46	287 ± 84	0.002	125 - 647
Ornithine	104 ± 37	32 ± 14	0.001	0 - 157
Total amino acids	1.76 ± 0.51	2.96 ± 0.60	0.002	
EAA	0.62 ± 0.16	1.13 ± 0.23	0.002	
% EAA	35 ± 2	38 ± 4	0.10	

DISCUSSION

In the majority of neonatal intensive care units amino acids are not administered to preterm infants from birth onwards, but supplementation is started later [2]. The resulting loss of protein mass will depend on the catabolic state of the infant and the time of commencement of the amino acid supplementation. The reason for the delay in introducing the amino acid administration is usually the concern about the diminished metabolic capacity of preterm newborns to handle an amino acid load. In utero however, the fetus receives amino acids [1]. Recent studies have shown no adverse effects of introducing iv amino acids as early as the first day of life [5,6,8], but potential side effects like elevated plasma amino acid levels or uremia, were only measured after a few days of life and never on the first postnatal day. During the first postnatal day, the metabolic side effects could be maximal. We have therefore performed the present study, measuring the effect of introducing amino acids within a few hours of birth on leucine kinetics, amino acid concentrations, pH and urea in ventilated very-low-birth weight infants on the first day of life.

With an amino acid intake of 1.15 $g.kg^{-1}.d^{-1}$, we did not observe serious side effects, while, on average, the infants who received amino acids were not in a negative nitrogen balance any more. The range in nitrogen excretion was however wide, probably reflecting the clinical condition. Blood urea level was not statistically significantly higher in the group who received glucose and amino acids, nor was the amount of administered bicarbonate (at similar pH and pCO_2) for the treatment of metabolic acidosis.

The amino acid solution, which we administered to the infants, contained cysteine-HCl. Laine *et al.* demonstrated that serum carbon dioxide was lower in premature infants who received total parenteral nutrition with the addition of cysteine-HCl than in infants who did not [22]. The infants in the present study received much less cysteine-HCl (180 $\mu mol.kg^{-1}.d^{-1}$) compared to the infants studied by Laine *et al.* (400 $\mu mol.kg^{-1}.d^{-1}$). This may explain why we did not observe a difference in acid-base status between the two groups.

Despite supplementation, plasma cystine level remained below the reference range, although we noticed a more than threefold increase in the group who received amino acids. We previously calculated by regression analysis that preterm infants need a cysteine intake of $\pm$ 500 $\mu mol.kg^{-1}.d^{-1}$ to obtain cysteine levels such as those found in growing normal breast fed infants [21,23].

These data were derived from one-week-old preterm infants on total

parenteral nutrition. The plasma cystine levels found in the present study following the administration of 1.15 g amino acids.$kg^{-1}.d^{-1}$ confirm a cysteine requirement of $\pm$ 500 μmol.$kg^{-1}.d^{-1}$. However, an effect on the acid-base status could be present at such an intake. A very interesting finding was that plasma cystine level was highly predictive for protein synthesis in the group receiving glucose and amino acids. Since all other amino acids were within the reference range, and no other amino acid could contribute to the prediction of the variation of protein synthesis, this finding might indicate that the plasma level of cystine was rate-limiting for protein synthesis in this group of infants. A suboptimal level of only one amino acid could have detrimental effects on protein synthesis. Johnson and Metcoff have shown that part of the variance in protein synthesis can be explained by plasma amino acids, but they found that intracellular amino acid levels are better predictors of protein synthesis [24]. However the explanation of the variance in protein synthesis in the present study is 75%, whereas the explanation found by Johnson and Metcoff was 23% for plasma levels and 36% for intracellular levels. The illness and size of the infants studied allowed us to take only small amounts of blood, so we were not able to measure intracellular amino acid levels in leukocytes.

In the 1970s, both Snyderman [25] and Pohlandt [26] showed that cyst(e)ine is an indispensable amino acid for the neonate. The importance of administering amino acid solutions with cysteine to preterm infants is also recently shown by Rivera *et al.*[8]. They found a higher nitrogen retention in infants receiving cysteine containing amino acid solutions compared to infants who received cysteine-free amino acid solution. Additional studies have to be carried out to evaluate the cysteine need of the preterm neonate.

In the past some concern was raised regarding toxic levels of phenylalanine in parenterally fed newborn babies. Although we found plasma phenylalanine levels slightly above the reference range in the supplemented group, the highest level was still less than 10% of the range found in classic phenylketonuria (> 1200 μmol/L) [27]. None of the other amino acids, except for aspartate, were above the reference level, also the total amount of amino acids was very close to that found in normal fetuses in the second and third trimester [1]. The infants who received exclusively glucose had lower total amino acid concentrations, with levels below the reference range for methionine, cystine, isoleucine and leucine, which are indispensable amino acids for the preterm neonate. Furthermore levels of arginine and glutamic acid were below the reference values. Suboptimal levels of amino acids might lead to suboptimal protein synthesis.

Table 4.5. Leucine kinetics in μmol.kg^{-1}.hr^{-1}.

	intake	turnover	recovery (%)	oxidation	NOD	LRP	balance
Glucose (n=8)	-	201 ± 20	20 ± 6	41 ± 13	160 ± 20	201 ± 20	-41 ± 13
Glucose and amino acids (n=7)	37 ± 2	217 ± 30	21 ± 8	46 ± 16	172 ± 32	180 ± 29	-8 ± 16†

† indicates a significant differences at $P = 0.01$.

By extrapolating the leucine turnover data to whole body protein turnover rates, it is possible to compare the turnover rates in these infants with the turnover rates in infants studied with the use of [^{15}N]glycine. Recently, we showed that protein turnover rates were 5-7 g.kg^{-1}.d^{-1} in three to four day old preterm infants [7]. Very similar results were found by Rivera *et al.*, using [1-^{13}C]leucine [8]. The turnover rates we found in the present study are higher, although the amino acid intake was lower. Although the turnover rates did not correlate with the ventilatory score, we hypothesize that the amount of stress caused by the birth, the clinical condition and the procedures in a neonatal intensive care unit are of such magnitude that the turnover is higher than found in three to four day old preterms. Stressed human adults have increased protein turnover rates as well [28,29].

We could not discriminate whether amino acid administration resulted in an inhibition of protein breakdown or in a stimulation of protein synthesis. Due to the relatively large standard deviation, the difference in the protein synthesis rate was not statistically significant. A sample size of at least 46 would be needed to detect that difference at the 5% level of significance on the basis of the variance of our data. However, both techniques used, [1-^{13}C]leucine turnover study and nitrogen retention data, which are indepedent, showed that there was a general improvement in nitrogen metabolism.

We found a significant effect with the administration of corticosteroids to mother just prior to birth on protein breakdown. Leucine turnover, leucine oxidation and leucine release from proteins are known to increase following steroid administration [17,30]. although we did not find an effect on leucine oxidation, it is interesting that the effect of steroids on protein breakdown is still measurable at least 24 hours after the corticosteroid administration.

Leucine oxidation was not increased by the supplementation of amino acids. The amount of energy derived from protein oxidation was 7-8 kcal.kg^{-1}.d^{-1} and was

not influenced by the administration of 1.15 g amino acids.$kg^{-1}.d^{-1}$. Energy expenditure of ventilated preterm infants is 40-50 kcal.$kg^{-1}.d^{-1}$ [31], and we have previously shown that glucose oxidation yields 17 kcal.$kg^{-1}.d^{-1}$ [12]. The remaining energy should be provided by other substrates such as fatty acids. Patel and Kalhan recently reported the oxidation rate of fatty acids in fasted term infants to be equivalent to 25 - 30 kcal.$kg^{-1}.d^{-1}$, measured by indirect calorimetry [32]. This would give the following energy partition: 35% - 40% is delivered by glucose oxidation, 15% - 20% is delivered by protein oxidation and the remaining energy needed ($\pm$ 50%) could be derived from fatty acid oxidation. Studies on fatty acid oxidation have to be performed in preterm infants to confirm this picture of energy partition.

It has been shown, in slightly older infants with a higher energy intake, that the anabolic effect of feeding on nitrogen metabolism is by stimulation of protein synthesis [7,8] and not by the suppression of protein breakdown. Stimulation of protein synthesis could be a direct effect of the amino acids, whereas the inhibition of proteolysis could result from a higher insulin level induced by the feeding. Glucose levels were however not statistically significantly different between the two groups, although the "G + AA" group had slightly lower glucose levels. It might well be that the effect of the amino acid administration is not as obvious as it would have been at a higher energy intake.

In conclusion we show that the administration of 1.15 g of amino acids directly from birth onwards improves average nitrogen retention even at energy intakes of less than 30 kcal.$kg^{-1}.d^{-1}$ in the relatively small group of infants we studied. Using [1-^{13}C]leucine as a tracer, it is not clear whether this effect is based upon stimulation of protein synthesis, inhibition of protein breakdown or a combination of both. We could not show a change in amino acid oxidation following amino acid administration.

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CHAPTER 5

AMINO ACID SOLUTIONS FOR PREMATURE INFANTS DURING THE FIRST WEEK OF LIFE: THE ROLE OF N-ACETYL-L-CYSTEINE AND N-ACETYL-L-TYROSINE.[36]

ABSTRACT

Tyrosine and cyst(e)ine are amino acids that are thought to be essential for preterm neonates. These amino acids have a low stability (cyst(e)ine) or solubility (tyrosine) and are therefore usually only present in small amounts in amino acid solutions.

Acetylation improves the stability and solubility of amino acids, facilitating a higher concentration in the solution. We compared three commercially available amino acid solutions; Aminovenös-N-päd 10%, Vaminolact 6.5% and Primène 10%, administered to 20 very-low-birth-weight infants on total parenteral nutrition from postnatal day two onwards. Aminovenös-N-päd 10% contains acetylated tyrosine (NAT) and cysteine (NAC), the other solutions do not contain acetylated amino acids and differ in the amount of tyrosine and cysteine added.On postnatal day 7, plasma amino acids were measured, together with urinary excretion of amino acids and the total nitrogen excretion.

38% of the intake of N-acetyl-L-Tyrosine (NAT) and 53% of the intake of N-acetyl-L-Cysteine (NAC) was excreted in urine. Plasma levels of NAT (331$\pm$74 μmol/L) and NAC (18$\pm$29 μmol/L) were higher than of tyrosine (105$\pm$108 μmol/L) and cystine (11$\pm$9 μmol/L), respectively. We found a linear correlation of plasma cystine with the intake of cysteine ($r=0.75$, $p=0.01$), but not with NAC. The estimated intake of cysteine should be $\pm$ 500 $\mu mol.kg^{-1}.d^{-1}$ in order to obtain levels comparable to those found in normal term breast-fed infants.

Plasma tyrosine levels in the groups receiving small amounts of tyrosine remained just below the reference range. Nitrogen retention was not different for the three groups (247 - 273 $mg.kg^{-1}.d^{-1}$).

We conclude that a large portion of parenterally administered acetylated amino acids is excreted in urine in one week old, preterm infants, with higher plasma levels of the acetylated amino acids than the deacetylated amino acids.

Abbreviations:

NAT = N-acetyl-L-Tyrosine
NAC = N-acetyl-L-Cysteine
EAA = Essential Amino Acids

INTRODUCTION

Limited tolerance of premature infants to early introduction of oral feeding necessitates the parenteral administration of nutrients. Total parenteral nutrition during the first week of life may reduce the risk of necrotizing enterocolitis [1-3] and enables the supply of energy and amino acids. In the past there have been concerns about the administration of protein hydrolysates and amino acid mixtures to premature infants, because of metabolic complications such as hyperammonemia, acidosis and very high levels of specific amino acids [4,5,6]. Recent cristalline solutions have shown a reduction of those metabolic risks and early administration of amino acids has been shown to increase nitrogen retention and to promote protein synthesis [7,8].

Controversy exists regarding the amount and manner of administration of amino acids such as tyrosine and cystine in pediatric amino acid solutions. Both amino acids are thought to be essential in the neonatal period [9-13], but they have a low solubility (tyrosine) or stability (cyst(e)ine). Acetylation increases the solubility and stability, facilitating a higher concentration in the amino acid solution.
However, the administration of NAT to premature infants did not result in levels within the reference range, with a large fraction being excreted in the urine [14]. No studies regarding NAC metabolism in preterm infants are available to date.

The purpose of our study was to compare three different amino acid solutions by measuring plasma amino acids in 7 day old preterm infants on total parenteral nutrition. The amino acid solutions differ especially in the amount and manner of administration of tyrosine and cystine. Special attention is given to these two amino acids.

PATIENTS

The infants in the study were admitted to our neonatal intensive care unit on their first day of life. Their birth weight was below 2000 g. Gestational age was assessed from the date of the last menstrual period, ultrasound data and the Ballard score [15]. All infants were clinically stable at the time of the study (postnatal day 7), with normal renal and liver functions. Patient characteristics are described in **Table 5.1.** On day one of life, the infants were randomly assigned to one of three groups to receive as amino acid solution either: group I Aminovenös-N-päd 10% (Fresenius AG, Bad Homburg, Germany), group II Vaminolact 6.5% (Kabi Pharmacia,

Table 5.1. Characteristics of the patients

	GROUP I Aminovenos n=10	GROUP II Vaminolact n=4	GROUP III Primene n=6
birth weight (kg)	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.2
study weight (kg)	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.2
gestational age (wk)	31 ± 2	30 ± 2	30 ± 2
Nutrient intake day 7			
Amino acids ($g.kg^{-1}.d^{-1}$)	2.3 ± 0.2	2.1 ± 0.2	2.2 ± 0.3
Total Energy ($kcal.kg^{-1}.d^{-1}$)	69 ± 8	60 ± 6	66 ± 6
Determinations day 7			
pH	7.33 ± 0.04	7.32 ± 0.00	7.29 ± 0.03
BE	-3.4 ± 2.5	-3.6 ± 1.4	-3.9 ± 2.0
bilirubin (mmol/L)	134 ± 29	106 ± 64	137 ± 29
phototherapy on day 7	10/10	2/4	4/6

Stockholm, Sweden) or group III Primène 10% (Clintec Benelux NV, Brussels, Belgium). No statistically significant differences were found in birthweight, weight at the time of the study or gestational age. Packed red blood cells were given to two infants (one of group I at day 3 and one of group III at day 6), but none of the infants received plasma or albumin for at least 72 hours prior to the study.

Informed consent from one or both of the parents was obtained prior to the study. The study was approved by the ethical committee of our hospital.

FEEDING REGIMEN

The infants did not receive enteral feedings during the first week of life, which is the feeding policy of our unit [1]. On day one of life, the infants received 4.2 mg $glucose.kg^{-1}.d^{-1}$ only. Amino acids and fat were gradually introduced on day two,

Table 5.2. Composition of the amino acid solutions (mg amino acid/g of mixture).

	Solution I Aminovenös-N-päd 10%	Solution II Vaminolact 6.5%	Solution III Primène 10%
Threonine†	49	55	37
Valine†	68	55	75
Cysteine/cystine†		15	24§
N-acetyl-cysteine†	5		
Methionine†	44	20	24
Isoleucine†	61	47	66
Leucine†	103	107	99
Tyrosine†		8	4
N-acetyl-tyrosine†	65		
Phenylalanine†	44	41	42
Tryptophan†	17	21	20
Lysine†	95*	86	109
Histidine†	40	32	38
Arginine†	61	63	83
Aspartic acid		63	60
Serine	86	58	40
Glutamic acid		109	99
Proline	155	86	30
Glycine	40	32	40
Alanine	68	96	79
Ornithine			25
Taurine		5	6
mol% EAA	56	48	55
g% EAA	65	55	62

† indicates essential amino acid for the preterm neonate, *Lysine as Lysine-monoacetate,
§ cysteine/cystine as cysteine-HCl.

with an aimed intake of 2.4 g amino acids.$kg^{-1}.d^{-1}$ and 2.4 g fat.$kg^{-1}.d^{-1}$ (Intralipid 20%, Kabi Pharmacia, Stockholm, Sweden) on day 3-7. Glucose intake was increased gradually to 8.3 mg.$kg^{-1}.d^{-1}$ on postnatal day 6, after which the intake remained unchanged. The daily intake was decided by the attending physician and was not influenced by the investigators.

The composition of the amino acid solutions as given by the manufacturers is shown in **Table 5.2.** No additional cysteine hydrochloride was thus added to the parenteral nutrition infusates before administration to the patients. All patients received the nutrients via a peripheral infusion.

METHODS

Heparinized blood samples were collected, either from an arterial line or by heel stick on day 7 of life. The blood samples were taken at a distance and retrograde from the site of amino acid infusion. The samples were immediately put on melting ice and centrifugated for 10 minutes at 5.000 *g* at 4° C within 10 minutes of their collection. The supernatant was stored at -80° C until analysis. Amino acid levels were determined on either a LKB 4151 Alpha plus, Amino Acid Analyser (Pharmacia LKB Biochrom Ltd., Cambridge, United Kingdom) or a LC 5000 Biotronic (Munich, Germany) [16]. A mean difference of 0.6 ± 3.6% [range -7.1% - 7.4%] was found at running an amino acid calibration standard (Pharmacia LKB Biochrom Ltd., Cambridge, United Kingdom) on both machines. NAT and NAC were determined by an HPLC method, as previously described [17,18]. A 24 hour urine collection with adhesive bags was performed to determine the nitrogen retention [8]. Urinary amino acids were determined using the same method as for the plasma amino acids, but being measured on the LKB AA only. The percentage of the amino acid retained was calculated by dividing the total urinary excretion by the intake and substract this number from 1, times 100%. Blood gas analyses were performed at 08.00 h on day seven of life.

STATISTICAL ANALYSIS

Data are presented as mean ± 1SD. Comparison between the groups was performed using one way analysis of variance (Kruskall-Wallis). Because of the multiple variables assessed on single samples, differences in amino acid concentrations were considered to be significant at $P \leq 0.01$.

RESULTS

Total amino acid and energy intake did not statistically differ between the three groups, although the energy intake tended to be lower in the Vaminolact 6.5% group as compared to the Aminovenös-N-päd 10% group and the Primène 10% group. We found no differences in pH, base excess or total bilirubin, nor was the amount of administered bicarbonate different during the first week of life or in the last two days prior to the study.

Nitrogen retention, calculated by substracting the total urinary nitrogen excretion from the nitrogen intake, revealed no differences between the groups (group I 253 $\pm$ 72 $mg.kg^{-1}.d^{-1}$, group II 270 $\pm$ 30 $mg.kg^{-1}.d^{-1}$ and group III 247 $\pm$ 28 $mg.kg^{-1}.d^{-1}$). The percentage of nitrogen that was retained was highest (79%) in the infants from group II who had the lowest amino acid intake compared to infants of group I (67%) and infants of group III (68%). The differences were not found to be statistically significant and independent of energy intake.

Plasma amino acid levels are shown in **Table 5.3**. Plasma amino acid levels of breast-fed, growing infants have been added as reference values [19]. The total amount of plasma amino acids was not different between the groups. The concentration of essential amino acids, not including NAT and NAC, was lowest on Aminovenös-N-päd 10%, so was the percentage essential amino acids of the total amount of amino acids ($P = 0.05$). Compared to amino acid levels of post-prandial breast-fed term infants, only a few of the amino acid levels were far out of the reference range. In group I, three amino acid levels were slightly above the reference range (threonine, proline and glycine), but cystine was very low. In group II, threonine was higher compared to the reference range, phenylalanine and glycine slightly higher, but tyrosine and cystine below the reference range. Group III showed only minor differences compared with the reference data, being higher for valine, lysine, glycine, ornithine and lower for tyrosine.

Table 5.4 focusses on tyrosine and cyst(e)ine metabolism. The administration of NAT (group I) resulted in tyrosine levels within the reference range, but the NAT levels were on average three times as high. Almost 40% of the intake of NAT was lost in urine. Smaller amounts of tyrosine were given in the other groups resulting in tyrosine levels just below the reference range. The low amount of administered NAC (group I) resulted in low values of plasma cystine. More than 50% of the administered NAC was nevertheless excreted in urine.

Linear regression analysis on plasma cystine levels and the intake of cysteine,

thus using the data from group II and III only, revealed the following equation:

$$cystine_{plasma}(\mu mol/L) = 0.08 \times cysteine_{intake}(\mu mol.kg^{-1}.d^{-1}) + 11$$

with $P = 0.01$ and $r = 0.75$. NAC intake was not related to plasma cystine concentration.

We found no relation between the amount of amino acid excreted in the urine and the plasma level. For most amino acids, more than 95% of the intake was retained, although the plasma levels were sometimes above the reference range. Glycine was the only amino acid of which more than 5% of the intake was excreted in urine (on average 10% in all groups).

DISCUSSION

Information given by amino acid levels have to be interpretated with caution. A high plasma level of an amino acid could be caused by an excessive intake or a low utilisation rate of that amino acid. A third option could be an increased release of amino acids from body proteins resulting from a high protein breakdown. A low plasma level of an amino acid can originate from insufficient intake or a high utilisation rate.

Low plasma levels of essential amino acids indicate a relative deficiency state and this might be detrimental to protein synthesis. It is however difficult to determine abnormal levels for preterm infants. There is no "golden standard" to refer to when one has to interpret amino acid levels. We chose the data given by Wu et al as a reference [19]. The infants they studied were growing, stable, term infants with a postnatal age of one month. Therefore they were likely to have amino acid patterns that were optimal for protein synthesis. Other amino acid patterns, for instance measured in umbilical cord blood, show similar or smaller 95% confidence intervals [20].

Cystine is believed to be a semi-essential amino acid for the newborn [9]. Although methionine can be a precursor for cystine it is generally believed that the trans-sulfuration pathway (enzyme: hepatic cystathionase) through which cystine is synthesized from methionine is absent or low in the fetus and newborn infant [12,13].

Table 5.3. Plasma amino acid levels of the three groups with a reference range of term breast-fed infants [19]. Values in μmol/L or, in cases of total amounts, mmol/L. Values are depicted in bold whenever they are out of the reference range. The fifth colum depicts whether there are groups that are statistically significant different in their median value at $P \leq 0.01$.

	Group I Aminovenös-N-päd 10%	Group II Vaminolact 6.5%	Group III Primène 10%	significantly different	Term infants
Threonine†	**297±159**	**392±128**	194±54	II vs III	70 - 197
Valine†	154±33	126±27	**232±37**	I,II vs III	88 - 222
Methionine†	46±13	**21± 2**	32±11	I vs II	22 - 50
Cystine†	**11± 9**	**22± 8**	39±11		35 - 69
Isoleucine†	49±11	34±10	72±15	I,II vs III	27 - 90
Leucine†	86±19	102±23	119±19	I vs III	53 - 169
Tyrosine†	105±108	**30±23**	**32±11**	II,III vs I	38 - 119
Phenylalanine†	71±14	**85±19**	65±11		22 - 72
Lysine†	100±35	159±53	**250±49**	I vs III	80 - 232
Histidine†	88±25	104±20	107±48		34 - 119
Arginine†	43±11	53±17	94±45		42 - 148
Aspartic acid	41±19	23±6	48±30		5 - 51
Serine	282±89	237±56	277±125		0 - 326
Aspartate	19±7	21±8	43±29		16 - 81
Glutamic acid	57±26	76±10	123±71		24 - 243
Glutamine	360±101	554±188	534±142	I vs III	142 - 851
Proline	**412±153**	250±68	137±10	II vs III	83 - 319
Glycine	**383±136**	**417±67**	**405±126**		77 - 376
Alanine	218±101	282±78	270±85		125 - 647
Ornithine	62±28	63±17	**187±64**	I,II vs III	0 - 157
Total amino acids	3.1±0.7	3.1±0.6	3.3±0.7		
EAA§	1.0±0.3	1.2±0.2	1.2±0.3		
mol% EAA§	34±4	38±3	38±4		

§ plasma NAT and plasma NAC are not included.

Cystathionase activity increases with postnatal age [21], but it is not yet certain at what age the activity is sufficient. Pohlandt for instance, found low levels of cystine in preterm and term infants during the first two days of life [9], whereas Zlotkin et al found normal levels in older preterm infants [22]. In another study, Zlotkin and Anderson showed that the enzyme activity of hepatic cystathionase on the first day of life was 15% of the control activity and at 3 days of life 23% [23]. The preterm infants in the present study were studied on day 7 of life and in 17 out of 20 infants, cystine levels were below the 95% confidence interval. The lowest values were found in group I, receiving Aminovenös-N-päd 10% with the acetylated cysteine, despite methionine levels in the upper normal range. This indicates that in this group of preterm infants studied on day 7 of life, the cystathionase activity is still not optimal. We also found a high correlation between cystine level and cysteine intake in the other two groups, again pointing at the semi-essentiality of the amino acid. Extrapolating our regression equation, we can calculate that almost 500 μmol cysteine.$kg^{-1}.d^{-1}$ should be administered to obtain a plasma cystine concentration of 52 μmol/L, which is the mean value of cystine in growing term infants [19].

The three amino acid solutions tested, differed in their way and amount of cyst(e)ine administration. Cysteine administered as cysteine HCL (administered to the infants of group III) is referred to affect the acid-base homeostasis [24]. We did not find a statistical difference of pH between group III (with addition of cysteine HCl) and the other groups, although the lowest pH was found in group III. Also the base excess and the amount of bicarbonate added to keep pH within normal levels was not statistically different. The plasma cystine levels of group III were within the 95% confidence interval.

The administration of the low amounts of NAC as given to the infants of group I did not result in sufficient cystine levels. The infants in group I had the lowest levels. Despite the low intake and the low plasma levels, we found that more than 50% of the administered NAC was excreted in urine, which is higher than found in human adults [25,26].

Tyrosine is a semi-essential amino acid for the preterm neonate and has a low solubility [10]. In solution I (Aminovenös-N-päd 10%, but also in for instance Trophamine [TA], McGaw Inc, USA), this problem has been circumvented by the addition of acetylated tyrosine to the solution. We found normal plasma tyrosine levels in the infants receiving solution I (Aminovenös-N-päd 10%), whereas the two other groups showed levels slightly below the reference value. However, plasma NAT levels were high in group I, sometimes resulting in levels above 300 μmol/L.

Table 5.4. Tyrosine and cyst(e)ine data of the three different group. Reference range for tyrosine is 38 - 119 μmol/L, for cystine 35 - 69 μmol/L [19].

	Aminovenös-N-päd 10%	Vaminolact 6.5%	Primène 10%	units
TYROSINE				
intake tyrosine	--	88 ± 8	55 ± 9	$\mu mol.kg^{-1}.d^{-1}$
intake NAT	695 ± 75	--	--	$\mu mol.kg^{-1}.d^{-1}$
plasma tyrosine	105 ±108	30 ± 23	32 ± 11	μmol/L
plasma NAT	331 ± 74	--	--	μmol/L
% NAT retained	62%	--	--	
CYSTINE				
intake cyst(e)ine	--	170 ± 10	345 ± 54	$\mu mol.kg^{-1}.d^{-1}$
intake NAC	83 ± 9	--	--	$\mu mol.kg^{-1}.d^{-1}$
plasma cystine	11 ± 9	22 ± 8	39 ± 11	μmol/L
plasma NAC	18 ± 29	--	--	μmol/L
% NAC retained	47%	--	--	

No data exist regarding the potentially harmful effects when acetylated amino acids are above the, for tyrosine, normal levels. Tyrosinaemia, however, is associated with detrimental long term effects [27,28]. As was the case with NAC, we found high urinary excretion rates of NAT (±40% of the intake), although lower than those found in parenterally fed adults [25,29], but higher as compared to adult rats [17]. The only other study on NAT metabolism in preterm infants by Heird et al also describes that 60% of the NAT is retained [14]. The intake of NAT in that paper is only 25% of the NAT intake of the patients in group I. Heird *et al.* found tyrosine levels below the reference value and, as in the present study, NAT levels

exceeding the tyrosine levels.

Since a relatively high percentage of NAT and NAC was excreted in the urine, it can be hypothesized that the deacetylation of those substrates is not optimal. NAC is being used as a mucolytic agent and as therapy for paracetamol intoxication, with satisfactory results [30,31]. In those applications NAC is usually administered orally and thereby brought in contact with the most potent hydrolysing tissue: the intestinal mucosa [32]. Both the hepatocyts and the renal cells are much less capable of hydrolysing NAC [25,32]. With the parenteral administration of the acetylated amino acids in the present study, the deacetylation will hardly occur in the intestinal mucosa. Another contribution to the poor hydrolysis could be a less well developed deacetylation enzyme system due to the prematurity of the neonates.

Although NAT has been reported to be efficiently utilized in the rat [17], the efficiency for humans is still under debate [14,25,29]. The acetylase activity might be higher in rats than in humans. Helms et al found low tyrosine levels in preterm infants fed a parenteral amino acid solution containing 70% of the tyrosine intake (total 227 μmol/(kg.d)) as NAT [33]. There is no indication of hepatic extraction and conversion of NAT to tyrosine in humans. NAC and NAT could be even competing for the same enzyme system, resulting in normal levels of tyrosine and low levels of cystine, since solution I (Aminovenös-N-päd 10%) contains eight times more NAT than NAC.

Hyperphenylalaninemia (> 600 μmol/L), a matter of concern in the past with a specific amino acid solution [6,34,35], has not been found in any of the patients studied, although the phenylalanine concentration was slightly above the reference value in Vaminolact 6.5% group. We found no correlation between the two aromatic amino acids, tyrosine and phenylalanine, indicating a variable conversion.

Our study shows that 40-50% of the administered acetylated amino acids is excreted in urine by preterm infants. Plasma levels of cystine remain far below the reference range when the intake only consists of N-acetyl-L-cysteine given in the low amounts as in this study. We found a linear relationship between cysteine intake, but not N-acetyl-L-cysteine, and the plasma concentration of cystine. At least 300 μmol.kg^{-1}.d^{-1} should be given theoretically to get cystine levels at the lower end of the 95% confidence interval, and about 500 μmol.kg^{-1}.d^{-1} to be in the middle of the reference range. With the administration of 700 μmol NAT.kg^{-1}.d^{-1}, plasma tyrosine levels are within the reference range, but plasma NAT levels are three times as high and clearly above the reference range for tyrosine. With the administration of 50 to 100 μmol tyrosine.kg^{-1}.d^{-1}, levels are below the reference range. Due to the

essentiality of tyrosine and cysteine in the neonatal period and their instability or insolubility, other forms of substitution have to be found, because low amino acid levels may have a detrimental effect on protein synthesis. A potentially interesting development could be the use of dipeptides to increase the amount of administered tyrosine and cysteine to the patients [17,29], although no study is available in preterm infants to date.

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CHAPTER 6

PROTEIN METABOLISM IN PRETERM INFANTS ON TOTAL PARENTERAL NUTRITION.[37]

ABSTRACT

The time of commencement with amino acid administration following birth and the chemical composition of the amino acid solution are both important issues in feeding the very-low-birth weight infant. We investigated on postnatal day 7: a) whether there was an effect on protein kinetics of starting amino acid administration within a few hours of birth compared to starting on postnatal day 2, and b) whether there was an effect on protein kinetics in two groups of infants receiving amino acid solutions with a different chemical composition, containing either acetylated amino acids or not. To calculate protein synthesis and protein breakdown rates, we infused [1-^{13}C]leucine as a tracer and measured the dilution of the intracellularly formed [1-^{13}C]keto-isocaproic acid. Combining these results with 24 hour urinary nitrogen excretion rates, we were able to calculate protein breakdown and synthesis rates.

Eight preterm infants (group I, birth weight 1.3 ± 0.4 kg, gestational age 30 ± 1 wk) receiving an amino acid solution (amino acid intake on day 7: 2.3 ± 0.2 $g.kg^{-1}.d^{-1}$) almost from birth onwards, were compared to eight infants (group II, birth weight 1.4 ± 0.3 kg, gestational age 30 ± 2 wk) receiving the same amino acid solution (2.2 ± 0.3 $g.kg^{-1}.d^{-1}$ on day 7) from postnatal day 2 onwards. Whole-body protein synthesis rates were not statistically different (8.2 ± 1.7 $g.kg^{-1}.d^{-1}$ in group I *vs.* 9.0 ± 0.7 $g.kg^{-1}.d^{-1}$ in group II) nor was the whole-body protein breakdown rate (6.7 ± 1.7 $g.kg^{-1}.d^{-1}$ in group I *vs.* 7.5 ± 0.7 $g.kg^{-1}.d^{-1}$ in group II). Accordingly, nitrogen retention was also not significantly different.

Seven preterm infants (group III, birth weight 1.4 ± 0.2 kg, gestational age 30 ± 2 wk) received an amino acid solution containing acetylated tyrosine, cysteine and lysine (2.0 ± 0.4 $g.kg^{-1}.d^{-1}$ amino acids on day 7) from postnatal day 2 onwards. Although whole-body protein synthesis rate (8.9 ± 1.1 $g.kg^{-1}.d^{-1}$) and whole-body protein breakdown rate (7.8 ± 1.2) was not significantly different from those of group II, nitrogen retention was significantly lower in the infants receiving the acetylated amino acids (245 ± 32 mg $N.kg^{-1}.d^{-1}$ in group II *vs.* 176 ± 58 mg $N.kg^{-1}.d^{-1}$ in group III, $P < 0.05$). Combining the data from all patients, we found a positive exponential relation of energy intake and protein synthesis.

We conclude that starting the amino acid administration within a few hours of birth does not have an effect on protein kinetics on day 7. The composition of the amino acid solution seems to have an effect on nitrogen retention although we did not find statistically significant differences in whole-body protein synthesis or breakdown rates.

INTRODUCTION

Very-low-birth weight infants experience a loss of body weight during the first few days of life, which in part is the result of inadequate nutrient supply. The introduction of oral feeding may be delayed because of intolerance and because a delay in the introduction might lower the incidence of necrotizing enterocolitis [1]. Parenteral nutrition is the alternative to supply the preterm infant with calories and amino acids.

Protein hydrolysates have been used in the past to supply the newborn with amino acids, but metabolic side effects as hyperammonemia and the fixed amino acid composition have prompted attempts to replace those solutions. Infants infused with early mixtures of crystalline amino acids often experienced acidosis and hyperammonemia [2-5]. Recently developed amino acid solutions have shown to be relatively safe for preterm infants [6-10].

Accordingly, the time of commencement of amino acid administration has shifted towards the first day of life [6,7,11] to minimize the loss of lean tissue and to stimulate protein deposition. The beneficial effect of early supplementation might not be limited to the first days of life, but may also enhance nitrogen retention later on. The supplementation of amino acids to adults within five hours after a burn injury resulted in higher nitrogen retention up to twenty days after the trauma compared to patients who received amino acids 55 hours after the trauma [12].

The composition of the amino acid solution may have a pronounced effect on protein metabolism [13]. The composition of the presently available amino acid solutions is however quite different from each other, especially regarding two semi-essential amino acids for the neonate; tyrosine and cyst(e)ine [14-18]. Due to the limited solubility and instability, it is only possible to dissolve very limited amounts of these two amino acids and hence difficult to supply the neonate adequately. Some amino acid solutions contain therefore acetylated tyrosine and cysteine, which are more stable and have a much higher solubility. Nevertheless, recent studies have shown that the utilization of acetylated amino acids might not be very high, with concomitantly low plasma concentrations of the de-acetylated amino acids [9,19]. The information obtained by measuring plasma amino acids is limited. Low levels of amino acids can originate from a low intake, high utilisation, limited release of amino acids from protein breakdown or, in case of a non-essential amino acid, low endogenous production. the determination of plasma amino acid levels does not give information on protein kinetics. It is possible to measure protein synthesis and

protein breakdown with labelled amino acids by stable isotope tracer dilution techniques.

The aim of this study was twofold. Firstly, we investigated whether we could detect a beneficial effect of immediate commencement of amino acid administration, so from birth onwards, on protein kinetics on day seven. Second, we investigated whether different amino acid solutions given to preterm infants from day 2 of life onwards would result in different protein kinetics (*i.e.* differences in protein synthesis or breakdown) measured on day seven of life. Rates for protein turnover were obtained by infusion of [1-^{13}C]leucine and measuring the enrichment in the keto-analogue of leucine; α-ketoisocaproic acid, which is formed intracellularly.

PATIENTS AND METHODS

Patients and feeding regimen. The infants of the study were admitted to our neonatal intensive care unit at their first day of life. Birth weight of the infants was below 2000 g. Gestational age was assessed from the date of the last menstrual period, ultrasound data and the Ballard score [20]. All infants were clinically stable at the time of the study (postnatal day 7), with normal renal and liver functions. Patient characteristics are described in **Table 6.1.**

On admittance, 23 infants were randomly assigned to three different groups. Group I received as amino acid solution Primène 10% (Clintec Benelux NV, Brussels, Belgium) starting immediately, so within a few hours of birth. Group II received Primène 10% from postnatal day 2 onwards, whereas group III received Aminovenös-N-päd 10% (Fresenius AG, Bad Homburg, Germany) from postnatal day 2 onwards. The amino acid composition of both solutions is given in **Table 6.2.** Aminovenös-N-päd 10% contains acetylated tyrosine and cysteine, whereas Primène 10% contains cysteine-HCl and tyrosine. All infants received 4.2 mg glucose.kg^{-1}.min^{-1} on day one of life. On the second day of life lipid administration was started (Intralipid 20%, Kabi Pharmacia, Stockholm, Sweden).

The aimed intake was 2.4 g amino acids.kg^{-1}.d^{-1} and 2.4 g fat.kg^{-1}.d^{-1} at day 3-7. Glucose intake was increased gradually to 8.3 mg.kg^{-1}.d^{-1} at postnatal day 6, after which the intake remained unchanged. The daily intake was decided by the attending physician and was not influenced by the investigators. No enteral feeding was given during the first week of life.

Informed consent from one or both of the parents was obtained prior to the study. The study was approved by the ethical committee of our hospital.

Table 6.1. Clinical characteristics of the infants. Weight gain is the measured over the first postnatal week.

	I	II	III
number of infants	8	8	7
birth weight (g)	1310 ± 364	1381 ± 253	1412 ± 211
gestational age (wk)	29.7 ± 1.0	30.3 ± 1.5	29.5 ± 1.5
study weight (g)	1277 ± 312	1350 ± 226	1364 ± 214
weight gain ($g.kg^{-1}.d^{-1}$)	-4.7 ± 10.3	-4.5 ± 14.0	-6.9 ± 11.3

Stable isotope administration. The infants received a primed continuous infusion of [1-^{13}C]leucine on day seven of life. The infusion lasted for three hours and was given *via* the same route as the nutrients were given. Priming dosage was 14 μmol/kg and preceded the continuous infusion of 0.24 $\mu mol.kg^{-1}.min^{-1}$. The concentration of [1-^{13}C]leucine was checked by an amino acid analyzer (LKB 4151 Alpha plus Amino Acid Analyzer, Pharmacia LKB Biochrom Ltd., Cambridge, United Kingdom) routinely used for quantitative analyses of amino acid levels. Blood samples were drawn before the start of the infusion, immediately put on melting ice and centrifuged, whereafter the plasma was stored at -70°C until analysis. The whole procedure did not exceed 15 minutes. The same procedure was followed after 150 minutes of [1-^{13}C]leucine infusion and 180 minutes of [1-^{13}C]-leucine infusion. A plateau in enrichment of plasma α-KICA is needed to perform steady state calculations. More blood samples are needed to provide information whether a plateau in α-KICA enrichment has been established, but we limited the amount of blood needed for research purposes for ethical reasons. Previous studies have shown that with the priming dose as was given in the present study, plateau in enrichment of plasma α-KICA can be achieved within two hours [21,22]. We therefore feel that with three hours of [1-^{13}C]leucine infusion and a priming dosage as given in the present study, steady state calculations can be used.

Urinary nitrogen excretion. 24 hour urine collections were performed to measure the nitrogen excretion. As previously described [23], we collected diapers for 24 hours. Nitrogen was eluted from the diapers with 175 mL mM citrate buffer. After freeze-

drying, the nitrogen content of the samples was measured in an automatic nitrogen analyzer (ANA 1400, Carlo Erba, Milan, Italy).

Plasma analyses. Leucine turnover was calculated using the reciprocal pool model [24,25]. Briefly, intracellular leucine is reversibly transaminated to its keto analogue; α-ketoisocaproate. The measurement of enrichment of α-KICA following [1-^{13}C]leucine infusion will therefore reflect the intracellular enrichment of leucine, which has shown to be correct [26,27].

One hundred μL of plasma was deproteinized by adding 250 μl sulfosalicylic acid (6% (w/v)), after the addition of the internal standards ketocaproic acid and ketovaleric acid. After keeping the samples for 5 min at 0°C, the samples were centrifuged and 150 μL 1,2-phenylenediamine (2 g%) dissolved in 6 M HCl was added to the supernatant. The tubes were heated at 100°C for 60 minutes and afterwards allowed to cool. After addition of 3 ml dichloromethane-hexane mixture (1:1, v/v) and mixing, the supernatant was evaporated to dryness under nitrogen at 50°C. Derivatization was performed by adding 50 μl *N,O-bis*(trimethylsilyl)trifluoro-acetamide after which the vials were immediately sealed under nitrogen and left at room temperature for 30 minutes.

Gas chromatography/mass spectrometry was carried out on a Hewlett Packard HP 5890 (Palo Alto, CA, USA) gas chromatograph coupled to a JEOL JMS-DX303 (Tokyo, Japan) mass spectrometer operating in the electron impact mode. Chromatography was carried out on a 25 m x 0.32 mm capillary column (CP-Sil5-CB, Chrompack BV, Middelburg, The Netherlands). Selective ion monitoring was carried out at m/z 232/233 for (m) and (m+1) respectively. The plasma α-KICA enrichments were determined using a calibration graph constructed from standard mixtures ranging from 0 to 20 mol% ^{13}C-α-KICA. The coefficient of variation for plasma α-KICA measurements was 2% (n=5). α-KICA enrichments were calculated as mol percent excess above baseline enrichment.

Calculations. Leucine turnover (Q_L in $\mu mol.kg^{-1}.hr^{-1}$) was calculated from the rise in plasma enrichment of α-KICA following [1-^{13}C]leucine infusion according to the equation:

$$Q_L = \left(\frac{IE_{infusate}}{IE_{plasma}} - 1\right) \times I_L$$

where $IE_{infusate}$ is the isotopic enrichment of the infusate, IE_{plasma} is the isotopic enrichment of α-KICA in plasma and I_L the infusion rate of [1-^{13}C]leucine in $\mu mol.kg^{-1}.hr^{-1}$.

Table 6.2. Composition of the amino acid solutions (mg amino acid/g of mixture).

	Aminovenös-N-päd 10%	Primène 10%
Threonine†	49	37
Valine†	68	75
Cysteine/cystine†		24§
N-acetyl-cysteine†	5	
Methionine†	44	24
Isoleucine†	61	66
Leucine†	103	99
Tyrosine†		4
N-acetyl-tyrosine†	65	
Phenylalanine†	44	42
Tryptophan†	17	20
Lysine†	95*	109
Histidine†	40	38
Arginine†	61	83
Aspartic acid		60
Serine	86	40
Glutamic acid		99
Proline	155	30
Glycine	40	40
Alanine	68	79
Ornithine		25
Taurine		6
mol% EAA	56	55
g% EAA	65	62

† indicates essential amino acid for the preterm neonate, *lysine as lysine-monoacetate, § cysteine/cystine as cysteine-HCl.

Table 6.3. Parenteral intake of the infants on postnatal day 7.

	I (n=8)	II (n=8)	III (n=7)
amino acids ($g.kg^{-1}.d^{-1}$)	2.27±0.24	2.17±0.30	2.03±0.36
glucose ($g.kg^{-1}.d^{-1}$)	9.6±2.4	9.4±1.8	10.0±1.3
fat ($g.kg^{-1}.d^{-1}$)	2.33±0.55	2.37±0.55	2.50±0.49
Energy ($kcal.kg^{-1}.d^{-1}$)	68.4±12.7	67.4±10.8	70.7±4.2

Leucine turnover is extrapolated to whole body protein turnover assuming that 1 gram of protein contains 590 μmol leucine [28]. Whole body protein synthesis and breakdown are calculated assuming that the body contains a well-mixed pool of nitrogen and that the pool size is constant throughout the study.Nitrogen leaving the pool should equal nitrogen entering the pool. Nitrogen can enter the pool *via* the diet (I) and *via* breakdown of proteins (B). Nitrogen used for synthesis of proteins (S) leaves this pool, as does nitrogen excreted in urine (E). In equation:

$$Q = S + E = B + I$$

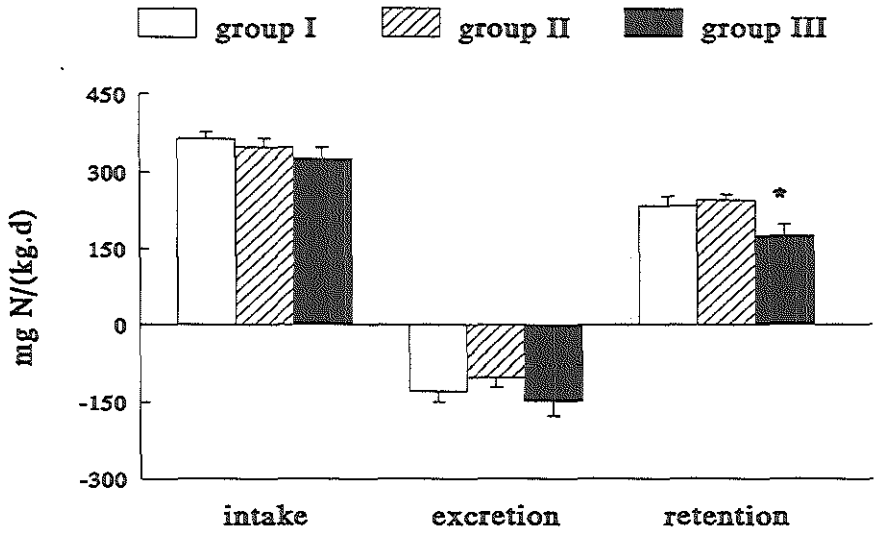
where Q is the whole-body protein turnover.

Statistics. Data are presented as mean ± 1SD. Differences between groups were considered to be statistically significant at a P value less than 0.05. A non-parametric test, Mann-Whitney-U, was used to detect differences between the two groups.

RESULTS

Birth weight, gestational age and weight at the time of the study was not significantly different for the three groups. On average all infants lost weight during the first week of life, as is shown in **Table 6.1.**

Figure 6.1. Nitrogen retention data. The asterix denotes a statistical significant difference between group II and III at a $P < 0.05$.



Comparison of starting amino acid administration from birth onwards or from postnatal day 2 (group I vs. group II).

We found no statistically significant differences in the intake of amino acids, glucose, fat or energy between group I and II on postnatal day 7 (**Table 6.3**). Nitrogen excretion was 129 ± 60 $mg.kg^{-1}.d^{-1}$ in group I and 102 ± 53 $mg.kg^{-1}.d^{-1}$ in group II.

Accordingly, nitrogen retention was also not significantly different (234± 50 $mg.kg^{-1}.d^{-1}$ group I *vs.* 245 ± 32 $mg.kg^{-1}.d^{-1}$ in group II). The utilization of the amino acid solution, expressed as the ratio of nitrogen retention and nitrogen intake x 100% was 65 ± 16% in group I and 72 ± 12% in group II.

Leucine turnover was not statistically different (220 ± 46 $\mu mol.kg^{-1}.hr^{-1}$ for group I and 237 ± 18 $\mu mol.kg^{-1}.hr^{-1}$ for group II). Whole-body protein kinetics, extrapolated from the leucine turnover rates, are shown in **Table 6.4**. Both protein synthesis and protein breakdown were lower in group I, but due to the relatively large standard deviation and small number of patients, those differences were not statistically significant.

The indices of protein kinetics were also quite similar. The efficiency of

nitrogen retention, expressed as the ratio of nitrogen retention and protein synthesis was 19 ± 7 % in group I and 17 ± 3 % in group II. The fraction of amino nitrogen flux used for protein synthesis, was 91 ± 4% in group I and 94 ± 3 % in group II.

Comparison of different amino acid solutions administered from postnatal day 2 onwards (group II vs. group III).

Neither the energy, nor the amino acid, glucose and fat intake was significantly different between group II and III on postnatal day 7 (**Table 6.3**). As can be seen in **Figure 6.1**, the difference in nitrogen excretion (102±53 $mg.kg^{-1}.d^{-1}$) group II *vs* 149 ± 78 $mg.kg^{-1}.d^{-1}$ group III) was not statistically significant, but nitrogen retention was higher in group II (245 ± 32 $mg.kg^{-1}.d^{-1}$ group II *vs.* 176 ± 58 $mg.kg^{-1}.d^{-1}$ group III, $P = 0.037$). The utilization of the amino solution in group III was 55 ± 17% compared to 72 ± 12% in group II, $P = 0.10$.

Leucine turnover rates were very much alike in both groups (237 ± 18 $\mu mol.kg^{-1}.hr^{-1}$ group II and 243 ± 23 $\mu mol.kg^{-1}.hr^{-1}$ group III). Concomitantly, whole-body protein turnover rates were not different either, since those rates are obtained from the leucine turnover data, **Table 6.4**. Whole-body protein breakdown rates were not significantly different (7.49 ± 0.71 $g.kg^{-1}.d^{-1}$ group II *vs.* 7.83 ± 1.18 $g.kg^{-1}.d^{-1}$ group III). Whole-body protein synthesis was very similar in both groups (9.03 ± 0.71 $g.kg^{-1}.d^{-1}$ group II *vs.* 8.94 ± 1.10 $g.kg^{-1}.d^{-1}$ group III).

The efficiency of nitrogen retention (retention/synthesis) was higher in group II, but the difference between the groups was not statistically significant (17 ± 3% group II *vs.* 13 ± 4 % group III, $P = 0.06$).

Since we did not find differences between group I and II and they both received the same amino acid solution, we compared the protein kinetics of the combination of the two groups with group III. The efficiency to retain nitrogen was higher in the infants receiving Primène 10% (18 ± 5% group I + II *vs.* 13 ± 4% group III, $P = 0.038$).

When taking the results of all patients together, we found a linear correlation between the weight loss during the first postnatal week and the protein breakdown rate ($r = 0.44$, $P = 0.038$). We did not find a correlation of protein kinetics and birth weight or gestational age. There was a significant relationship between protein synthesis rates and energy intake, which fitted best at an exponential function (**Fig 6.2**)

DISCUSSION

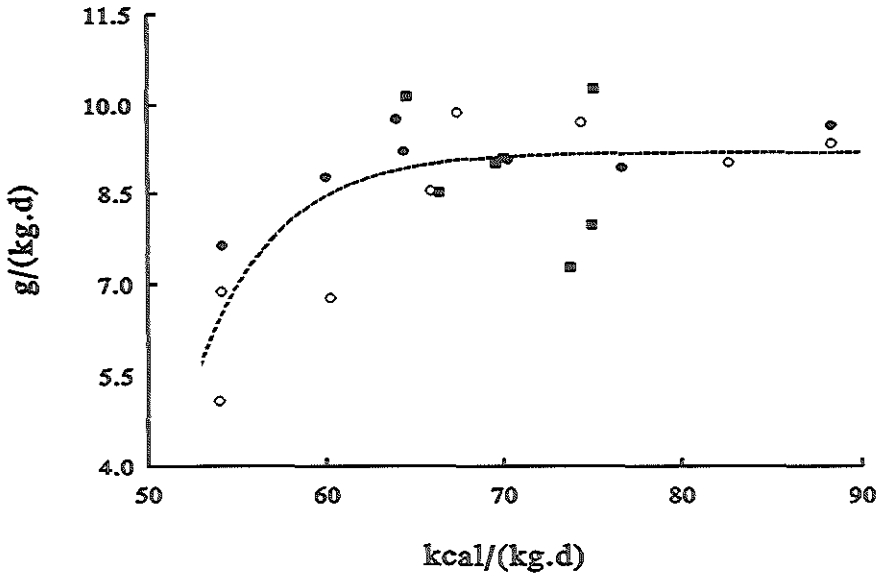
We have studied kinetics of protein metabolism, together with nitrogen balances in parenterally fed preterm infants at a postnatal age of one week. We had two questions, the first being whether we could find a beneficial effect of amino acid administration from birth onwards on protein kinetics on day seven of life. We could not find an effect of early supplementation in these preterm infants.

Previously, we have measured on postnatal day one the effect of early supplementation [11]. There is a clear positive effect of supplementation on amino acid retention on day one (both leucine retention, measured by [1-^{13}C]leucine dilution technique, and nitrogen retention were less negative), but we did not perform any protein turnover studies between postnatal day one and seven. The effect on nitrogen retention might be limited to one day. However, in burned adult patients, the timing of the commencement with supplementation (within 5 hr compared to 55 hr) seems to be important [12]. Nitrogen retention became significantly higher on 8-20 days after the injury. Saini *et al.* measured serial nitrogen balances in two groups of preterm infants for 10 days, either starting within 24 hours of birth or after 72 hours [7]. They found significant differences in nitrogen retention during the first three days. There were no differences by 4-10 days, which is in agreement with the present study.

The second question was whether there was an effect of the administration of rather different amino acid solutions to preterm infants on protein kinetics. A major difference between the two amino acid solutions is the chemical form in which tyrosine, cysteine and also lysine are being supplied. Tyrosine and cysteine are essential for the preterm infant, but not for adults. Previously we have shown that plasma cystine concentration was very low in preterm infants who received Aminovenös-N-päd 10% for one week [19]. On day one of life, we showed that if the mean plasma level of cystine was the only amino acid below the reference range, the plasma level of cystine closely related to protein synthesis ($r=0.83$) [11]. Rivera *et al.* have shown that cysteine supplementation resulted in an improved nitrogen balance of preterm infants [29]. However, others have found no effect cysteine supplementation in slightly older and larger infants [30,31].

In the present study we show that the nitrogen retention is lower in infants who received Aminovenös-N-päd 10%, containing small amounts of acetylated cysteine. Unfortunately we were not able to measure amino acid levels in these infants, to correlate plasma levels with protein kinetics.

Figure 6.2. Relation of protein synthesis with energy intake. The dotted line represents the exponential relation ($y = 9.20 - 4.02 \times 10^5 \times e^{-0.22x}$) of energy intake and protein synthesis. Open circles: group I, closed circles: group II, closed squares: group III.



It is not yet clear how a deficiency of one amino acid influences protein kinetics. We could not demonstrate a statistically significant difference in either protein synthesis or breakdown between groups II and III. Nevertheless, the difference in nitrogen retention could almost completely (78%) be explained by the difference in protein breakdown rates. An increased protein breakdown will lead to an increased release of amino acids including cyst(e)ine, to provide the necessary amount and balance of amino acids for protein biosynthesis. The increase in protein breakdown seems in contradiction to our earlier findings [10] and also to the findings of Rivera *et al.*, where an increase in protein synthesis was observed after the administration of amino acids [29]. The protein synthesis rates found in the present study are among the highest found in parenterally fed preterm infants [13,21,22,29]. We speculate that the infants were already at their maximal synthesizing capacity in the present circumstances and could therefore only improve their nitrogen retention by lowering the breakdown rates. Duffy *et al.* also found that at protein synthesis rates of

Table 6.4. Whole-body protein kinetics. Data are expressed in $g.kg^{-1}.d^{-1}$.

	I (n=8)	II (n=8)	III (n=7)
Turnover	8.97 ± 1.88	9.66 ± 0.72	9.87 ± 0.93
Synthesis	8.16 ± 1.72	9.03 ± 0.71	8.94 ± 1.10
Breakdown	6.70 ± 1.74	7.49 ± 0.71	7.83 ± 1.18

7-9 $g.kg^{-1}.d^{-1}$, nitrogen balance improved by lowering the protein breakdown rates, while protein synthesis rates were not affected in infants receiving either a protein hydrolysate or a solution of crystalline amino acids [13]. It is possible to measure higher protein synthesis rates in *e.g.* orally fed preterm infants, but this is probably due to the increased activity of the digestive system.

The choice of a tracer might influence the results, because different tracers are metabolized in different tissues. For example, phenylalanine and lysine, commonly used amino acid tracers, are completely metabolized within the liver, whereas leucine is also metabolized in muscle. Whole-body protein kinetics, extrapolated from the kinetics of one amino acid should therefore be interpreted with caution. It is possible that other tracers yield different results, also in the present study.

Energy intake is one of the factors that influence protein synthesis rate. In the present study we found a relation between protein synthesis and energy intake (**Fig 6.2**). The findings of Duffy *et al.* support this result [13]. They reported that the energy intake had a significant effect on the fraction of N-flux utilized for protein synthesis. The protein-energy relation is not surprising because the synthesis of protein is an energy consuming process (1 - 2 kcal/gram protein [32,33]). Also Heird and Kashyap have found a direct relation between energy intake and nitrogen retention in orally fed preterm infants [34]. We found energy intake to be exponentially related to protein synthesis. This indicates that at low energy intake, protein synthesis might be impaired. Increasing energy intake above 80 $kcal.kg^{-1}.d^{-1}$ does not seem to lead to higher protein synthesis rates at this amino acid intake and under these circumstances. However, energy intakes above 80 $kcal.kg^{-1}.d^{-1}$ might enhance protein synthesis further if the amount of amino acids is increased.

In conclusion we can state that the composition of an amino acid solution

seems to have an effect on nitrogen retention. We found no effect of administering different amino acid solutions to preterm infants on protein kinetics, which might be due to the sensitivity of the method used. Furthermore, we could not show an effect of starting amino acid administration either on postnatal day 1 or day 2 on protein kinetics measured at one week of age.

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CHAPTER 7

THE EFFECT OF DEXAMETHASONE ON PROTEIN METABOLISM IN INFANTS WITH BRONCHOPULMONARY DYSPLASIA.[43]

ABSTRACT

Corticosteroids are increasingly used for the management of bronchopulmonary dysplasia in very-low-birth weight infants. Corticosteroids are known to result in protein wasting in human adults and rats. To determine to what extent this therapy affects protein metabolism in preterm infants, ten very-low-birth weight infants were studied: before a gradually tapered dexamethasone therapy started, at day 4 of treatment (dexamethasone dosage 0.35 ± 0.09 $mg.kg^{-1}.d^{-1}$) and in seven infants at day 19 of treatment (dexamethasone dosage 0.10 ± 0.01 $mg.kg^{-1}.d^{-1}$).

Protein breakdown and turnover rates were increased at day 4 of treatment, but not any more at day 19 of treatment. Protein synthesis rate was not significantly affected during dexamethasone therapy. Weight gain was severely diminished during the first week of treatment, but not during the two consecutive weeks.
We conclude that nitrogen balance during high dosages of dexamethasone is significantly lower due to an increase in proteolysis and not by a suppression of synthesis.

Abbreviations used:

α-KICA	α-keto-isocaproic acid
BPD	bronchopulmonary dysplasia
VLBW	very-low-birth weight

INTRODUCTION

Despite unwanted effects as hypertension [1-3], gastrointestinal complications [4] and myocardial hypertrophy [5], glucocorticosteroids are increasingly used in the management of bronchopulmonary dysplasia (BPD) in preterm infants [2,6-9]. The effect of corticosteroids on protein metabolism has been studied in detail in rats [*eg.* 10-14] and human adults [15-20] but hardly in preterm infants. Protein wasting is described for both rats and human adults, either caused by decreased rate of protein synthesis or an increased rate of protein breakdown or both. Very recently, studies of corticosteroid treated very-low-birth weight (VLBW) infants have appeared, showing increased blood urea levels and urinary nitrogen excretion [21] and increased plasma amino acid levels [22]. Specific amino acids labelled with stable isotopes, especially [^{15}N]glycine and [1-^{13}C]leucine, have been used to gain insight

in the kinetics of protein metabolism. In adults in a fed state, an increase in amino acid oxidation (+60%) and turnover(+15%) following glucocorticosteroid treatment has been reported using [1-^{13}C]leucine [15]. Protein turnover rates in preterm infants, measured with labelled amino acids, are much higher as compared to adults. We were concerned that changes of similar magnitude as found in adults on corticosteroids would result in very high protein oxidation and turnover rates in preterm infants.

The aim of the study was to investigate the effect of dexamethasone therapy for bronchopulmonary dysplasia on protein metabolism in preterm infants. Studies were performed before treatment and during a gradually tapered dexamethasone therapy at day 4 and day 19. Day 19 was included because evidence from animal studies suggest that the initial proteolytic effect of corticosteroids is not persisting [14].

PATIENTS AND METHODS

Informed consent was obtained from the parents of 10 infants, before the start of the dexamethasone therapy. The infants had been mechanically ventilated from birth. The decision to start the dexamethasone treatment was taken by the responsible clinician, because the radiografic abnormalities suggested chronic lung disease and conventional respirator weaning techniques had failed. Dexamethasone treatment consisted of a course adapted from Avery *et al.* [2], with a starting dose of 0.5 $mg.kg^{-1}.d^{-1}$ during the first three days and thereafter gradually tapered in 23 days. Patients were studied prior to the start of the dexamethasone treatment, at day 4 and at day 19 after the start of the therapy. If an unexplained setback in respiratory settings occurred during the tapering phase, a brief continuation of the high dosage or a return to higher dosage was given.

The infants were scored according to an adapted ventilatory scoring system [23,24] (**Table 7.1**). This ventilatory scoring system uses the concentration of oxygen in the inspiratory air (FiO_2), the mean airway pressure (MAP) and ventilatory frequency (VF) according to the following formula:

$$VS = VF \times MAP \times FiO_2$$

where VS is the ventilatory score. Before starting dexamethasone therapy, three infants had mild respiratory distress ($VS \leq 150$); four infants suffered from moderate respiratory distress ($150 < VS \leq 500$), while three infants had severe respiratory

distress (VS > 500). After 4 days of treatment only one infant had severe respiratory distress and one infant moderate. After 19 days, none of the infants suffered from severe respiratory distress.

The infants were orally fed, receiving the same formula (Nenatal™, Nutricia, Zoetermeer, The Netherlands), except one infant (#2) who received Nutrilon™ (Nutricia, Zoetermeer, The Netherlands). Type of formula remained the same for each infant throughout the study period. Total nitrogen and energy intakes were calculated from manufacturers' compositional data and the daily recorded intake of the formula. Energy and protein intake are shown in **Table 7.2.**

Weight was measured daily on an electronic scale with a resolution of 5 g. Weight gain was calculated per week and expressed in grams per kilogram per day.

Protein turnover studies. Leucine turnover studies were performed by parenteral administration of [1-^{13}C]leucine. Three of the infants received the tracer enterally at day 19 of the therapy because they had no i.v. line present. Leucine turnover (Q_L) was calculated according to the following equation:

$$Q_L = \left(\frac{IE_{infusate}}{IE_{plasma}} - 1 \right) \times I_L .$$

where $IE_{infusate}$ is the isotopic enrichment of the infusate, IE_{plasma} is the isotopic enrichment of α-KICA in plasma and I_L the infusion rate of [1-^{13}C]leucine in μmol.kg^{-1}.hr^{-1}. Leucine turnover is extrapolated to whole body protein turnover assuming that 1 gram of protein contains 590 μmol leucine. Whole body protein synthesis and breakdown are calculated assuming that the body consist of homogenous pool of nitrogen and that the size of pool is constant throughout the study. Nitrogen leaving the pool should equal nitrogen entering the pool. Nitrogen can enter the pool *via* the diet (I) and *via* breakdown of proteins (B). Nitrogen that is used for synthesis of proteins (S) leaves this pool, as does nitrogen that is excreted in urine (E). In equation:

$$Q = S + E = B + I$$

where Q is the whole-body protein turnover.

Leucine oxidation studies were performed as has been described for glucose oxidation studies previously [24]. Substrate oxidation rates are normally calculated by multiplication of the rate of disappearance of substrate times the recovery of carbon label following administration of carbon labelled substrate. The recovery of

Table 7.1. Study weight (SW), postnatal age (PNA), dexamethasone intake (DEXA in $mg.kg^{-1}.d^{-1}$) and the ventilatory score (VS). No ventilatory score is given if the patient was not mechanically ventilated.

	before dexa treatment				1st study during dexa				2nd study during dexa			
	SW	PNA	DEXA	VS	SW	PNA	DEXA	VS	SW	PNA	DEXA	VS
1	0.94	18	0	1550	0.97	22	0.49	513	1.16	35	0.10	65
2	2.545	52	0	-	2.605	56	0.31	-	2.98	69	0.10	-
3	1.735	33	0	26	1.62	37	0.30	-	1.94	50	0.10	-
4	0.92	18	0	478	0.97	26	0.31	168	1.35	52	0.10	-
5	1.505	27	0	591	1.48	33	0.27	29	1.74	44	0.09	-
6	1.5	30	0	272	1.48	36	0.30	23	1.675	50	0.12	-
7	1.375	26	0	375	1.51	36	0.30	-				
8	1.27	22	0	367	1.13	27	0.53	94	1.275	41	0.12	-
9	0.99	30	0	50	1.025	36	0.29	-				
10	1.1	21	0	783	1.145	25	0.39	67				
mean	1.4	28	0	449	1.4	33	0.35	89	1.7	49	0.10	9

label depends both on the CO_2 enrichment and the CO_2 production rate. With the administration of carbon labelled bicarbonate before the administration of carbon labelled substrate and the measurement of the enrichment of expired CO_2 during both bicarbonate and substrate infusions, it is possible to calculate the substrate oxidation rate without measuring the CO_2 production rate. Mechanical ventilation is however obligatory to perform oxidation studies with this technique because of our method of CO_2 collection.

[1-^{13}C]leucine (99% enriched, Isotec, Miamisburg, Ohio, USA) and $NaH^{13}CO_3$ (98.7% enriched, Isotec, Miamisburg, Ohio, USA) were diluted in 0.9% NaCl by the hospital's pharmacy. Both solutions were filtered through a 0.22 μ Millipore filter and put into 5 ml sterile vials. [1-^{13}C]leucine concentration in the infusate was measured quantitatively by an amino acid analyzer. $NaH^{13}CO_3$ enrichment in the infusate was checked by isotope ratio mass spectrometry (VG SIRA 10, Fisons Instruments, Isotech Lt., Middlewich, Cheshire, UK).

A priming dose of 6.9 μmol $NaH^{13}CO_3$/kg was followed by a continuous administration of 4.7 $\mu mol.kg^{-1}.hr^{-1}$. The administration lasted for two hours and was followed by a [1-^{13}C]leucine priming of 2 mg/kg. Continuous administration of [1-^{13}C]leucine (2 $mg.kg^{-1}.hr^{-1}$) thereafter, lasted for 3 hours. Tracers were given using a Harvard infusion pump (M22, Harvard Apparatus Co Inc S. Natick, MA, USA). The priming dose was administered to prime the bicarbonate pool in the body in order to reach a plateau in expiratory air sooner. If we were not able to perform an oxidation study, because the infant did not have an endotracheal tube placed, we administered the [1-^{13}C]leucine only. Blood samples were drawn before the administration of the stable isotopes, at 30 minutes before the end and at the end of the [1-^{13}C]leucine administration.

Expired CO_2 was collected four-six times before the start of the stable isotope study and at every 10 minutes during the second hour of the $NaH^{13}CO_3$ infusion. CO_2 collections were made after one hour of the [1-^{13}C]leucine infusion and seven times during the last hour of the [1-^{13}C]leucine infusion. Enrichment of CO_2 was measured on the isotope ratio mass spectrometer, as previously described [25].

Leucine kinetics were calculated according to the following equation:

$$Q_L = Int_L + LRP = Ox + NOD$$

where Q_L is the leucine turnover, Int_L is the leucine intake, LRP the rate of release of leucine from proteins, Ox the leucine oxidation and NOD the nonoxidative

leucine disposal. The latter is a measure of protein synthesis, whereas LRP is indicative for protein breakdown.

Plasma ^{13}C-α-KICA determination. Leucine is reversibly transaminated to its keto-analogue: α-keto-isocaproic-acid (α-KICA) within the cell [26]. Measurement of the enrichment of α-KICA following [1-^{13}C]leucine infusion reflects thus the intracellular enrichment of leucine, which is the site of incorporation of leucine in proteins as well as the site for decarboxylation of α-KICA to isovaleryl-CoA. We measured therefore the dilution of ^{13}C-α-KICA in plasma, in stead of [1-^{13}C]leucine.

100 μL of plasma was deproteinized by adding 250 μl sulfosalicylic acid (6% (w/v)), after the addition of the internal standards ketocaproic acid and ketovaleric acid. After keeping the samples for 5 min at 0°C, the samples were centrifugated and 150 μL 1,2-phenylenediamine (2 g%) dissolved in 6 M HCL was added to the supernatant. The tubes were heated at 100°C for 60 minutes and afterwards allowed to cool. After addition of 3 ml dichloromethane-hexane mixture (1:1, v/v) and mixing, the supernatant was evaporated to dryness under nitrogen at 50°C. Derivatisation was performed by adding 50 μl *N,O-bis*(trimethylsilyl)trifluoro-acetamide after which the vials were immediately sealed under nitrogen and left at room temperature for 30 minutes.

Gas chromatography/mass spectrometry was carried out on a Hewlett Packard HP 5890 (Palo Alto, CA, USA) gas chromatograph coupled to a JEOL JMS-DX303 (Tokyo, Japan) mass spectrometer operating in the electron impact mode. Chromatography was carried out on a 25 m x 0.32 mm capillary column (CP-Sil5-CB, Chrompack BV, Middelburg, The Netherlands). Selective ion monitoring was carried out at m/z 232/233 for (m) and (m+1) respectively. The plasma α-KICA enrichments were determined using a calibration graph constructed from standard mixtures ranging from 0 to 20 mol% ^{13}C-α-KICA. The coefficient of variation for plasma α-KICA measurements was 2% (n=5). α-KICA enrichments were calculated as mol percent excess above baseline enrichment.

Urinary nitrogen. An alternative method was used to collect urine for nitrogen balance data. Techniques using adhesive bags or condoms give variable results in accuracy of collection of the total urine production in VLBW infants and are usually only effective in boys. We collected diapers (Pampers, Procter and Gamble, Vlaardingen, The Netherlands) for 24 hours. The diapers were changed at least every three hours. Diapers that contained considerable amounts of faeces were not included. Diapers were put in separate plastic bags and stored at -20°C until

Table 7.2. Protein and energy intake of the infants during the study days.

	before	day 4	day 19
Protein ($g.kg^{-1}.d^{-1}$)	3.0 ± 0.4	3.3 ± 0.4	3.2 ± 0.2
Energy ($kcal.kg^{-1}.d^{-1}$)	114 ± 15	123 ± 16	119 ± 7

analysis. Upon analysis, each diaper was put in a glass cylinder and 175 ml 2 mM citrate buffer (pH=5.0) was added. The diapers were soaked with the buffer and mixed thoroughly on a rotating wheel for 2 hours at 5°C. A fixed sample was taken out of each cylinder. Samples from each diaper of the collection period were put together and after mixing and freeze drying, the total nitrogen content was measured on an automatic nitrogen analyzer (ANA 1400, Carlo Erba, Milan, Italy). Validation of this technique was performed using pooled urine. Nitrogen in the urine was measured directly and after pouring urine into diapers after which the diapers were treated as described above. Recovery percentages were >95% (n=10). Nitrogen excretion, other than via the urine, was estimated to be 10% of the nitrogen intake [27-30].

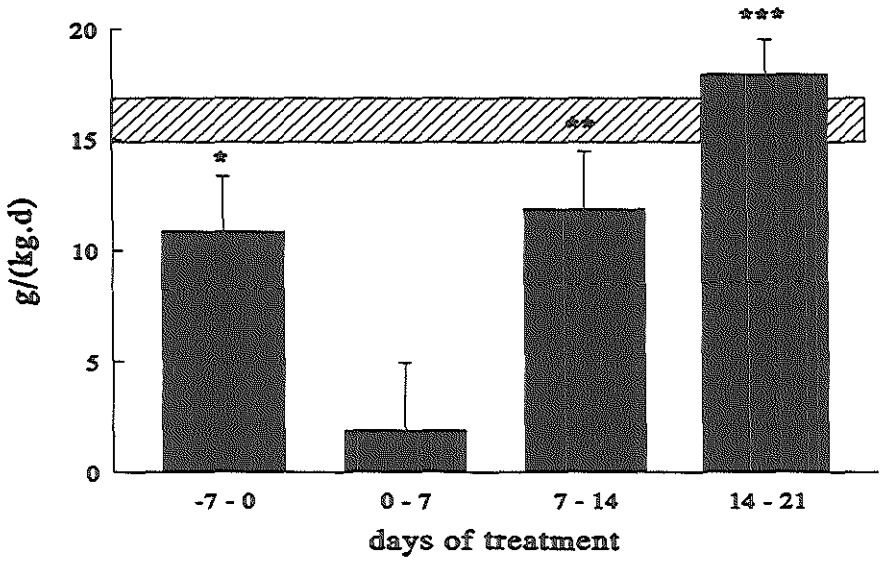
STATISTICAL ANALYSES

All data are presented as mean ± 1SD, unless otherwise stated. Statistical analysis was performed using non-parametric tests, first by using Friedman test to investigate whether there were differences between any of the three study days. If so, Wilcoxon matched pairs signed rank tests were performed to detect differences between two study days. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Average birth weight of the infants was 1.1 ± 0.3 kg, with a gestational age of 27.5 ± 2.0 wk. The infants started with dexamethasone on an average postnatal age of 29 ± 11 d. Postnatal age, study weight, dexamethasone intake and ventilatory score for the individual infants at the different study days are shown in **Table 7.2.** Leucine turnover studies were performed in 10 infants at day 0 and day 4 of treatment and in 7 infants at day 19 of the therapy. The infants studied on day 19 were either for the second or third day on 0.1 $mg.kg^{-1}.d^{-1}$ dexamethasone. One of the infants (#4) started at day 16 with a higher dosage (0.26 $mg.kg^{-1}.d^{-1}$ dexamethasone), while the

Figure 7.1. Weight gain in $g.kg^{-1}.d^{-1}$ during the week before treatment, and in the following 3 weeks. The hatched horizontal bar shows the intra-uterine weight gain. A difference in weight gain at the first week of the treatment at $P \leq 0.05$ by *, at $P \leq 0.01$ by **, and at $P \leq 0.005$ by ***.



dosage at day 15 was 0.15 $mg.kg^{-1}.d^{-1}$ dexamethasone. The course was gradually tapered from then onwards. In order to study the infant at the time she was receiving 0.1 $mg.kg^{-1}.d^{-1}$, we waited for 30 days after the dexamethasone was initially started.

Weight gain, measured during the week before the start and in the consecutive three weeks of the therapy is shown in **Figure 7.1.** The weight gain of the infants before the start of dexamethasone and during the first two weeks of dexamethasone therapy was lower compared to the calculated intra-uterine weight gain obtained from the 50^{th} percentile (15-17 $g.kg^{-1}.d^{-1}$) [31].

Nitrogen intake, urinary excretion and nitrogen retention are shown in **Figure 7.2.** Nitrogen excretion was positively correlated with dexamethasone intake (r=0.69, Spearman rank correlation test). The nitrogen retention was negatively correlated with the dexamethasone dosage (r=-0.57).

Whole-body protein turnover, breakdown and synthesis rates are shown in **Table 7.3.** Whole-body protein turnover rate was higher on day 4 of treatment

Table 7.3. Protein kinetics ($g.kg^{-1}.d^{-1}$) before and during the dexamethasone course. § indicates a statistical significant difference from "before" values at $P \leq 0.05$.

	before n=10	day 4 n=10	day 19 n=7
Turnover	12.3 ± 2.1	15.3 ± 3.4§	14.4 ± 2.8
Breakdown	9.3 ± 2.2	12.0 ± 3.1§	11.2 ± 2.8
Synthesis	11.2 ± 2.0	12.7 ± 3.7	12.9 ± 3.1

compared to the rate before treatment started (P=0.047). At low dexamethasone dosages, turnover rate was not significantly different from the initial value (P=0.18). Whole-body protein breakdown rates were significantly higher as well on day 4 (P=0.047) and not on day 19 (P=0.39). Protein synthesis was not significantly affected by the treatment.

Nine out of ten patients showed an increase of leucine turnover at day 4, with an average increment of 29% (302±52 $\mu mol.kg^{-1}.hr^{-1}$ before treatment started *vs.* 376±85 $\mu mol.kg^{-1}.hr^{-1}$ on day 4, P=0.046). At day 19, no statistically significant difference was found as compared to before treatment -(354±69 $\mu mol.kg^{-1}.hr^{-1}$, P=0.18).

The release of leucine from protein, (an indication of protein breakdown) increased by 42% at day 4 (207±54 $\mu mol.kg^{-1}.hr^{-1}$ before treatment started *vs.* 272±75 $\mu mol.kg^{-1}.hr^{-1}$ on day 4, P=0.01) and tended to be higher at day 19 as compared to before treatment (+18%, 251 ± 69 $\mu mol.kg^{-1}.hr^{-1}$, P=0.06).

Oxidation data before the start of the treatment are available for 7 patients only, due to technical reasons. Five infants still needed ventilatory support at day 4 of the treatment, so we could perform oxidation studies in those five infants. Plateaus in enrichment of expired CO_2 was reached within one hour of the labelled bicarbonate infusion and within two hours of the labelled leucine infusion. Leucine oxidation in the five patients studied at day 4 tended to increase from 53 ± 10 $\mu mol.kg^{-1}.hr^{-1}$ to 104 ± 30 $\mu mol.kg^{-1}.hr^{-1}$, with an increase in oxidation by 4 of the 5 patients (P=0.08).

Non-oxidative disposal, that reflects the amount of leucine that is being used

for protein synthesis, did not increase statistically significant at day 4 (240±40 μmol.kg^{-1}.hr^{-1} at day 0 *vs.* 303±87 μmol.kg^{-1}.hr^{-1} at day 4, P=0.14).

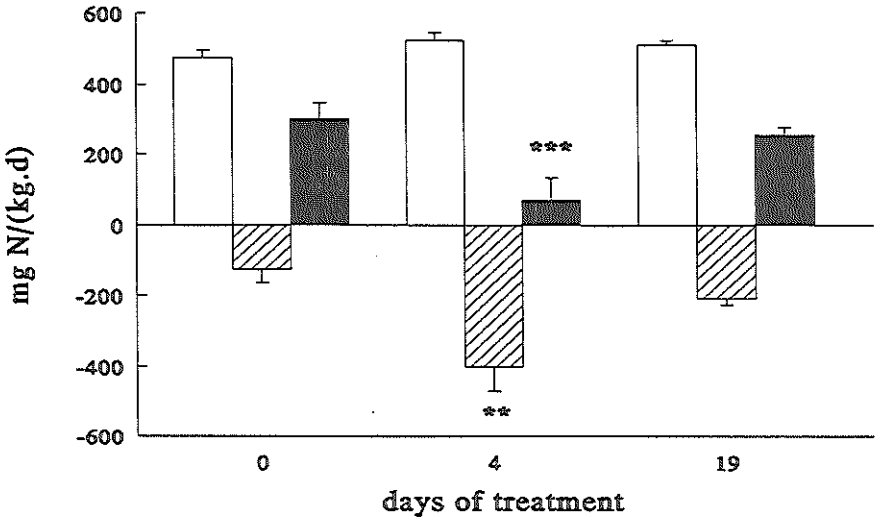
DISCUSSION

We have shown in this study that high dosages of dexamethasone, given on day 4 of a gradually tapered course decrease weight gain and increase urinary nitrogen excretion. Protein turnover is higher through a higher protein breakdown, whereas protein synthesis is not significantly affected in VLBW infants with BPD treated with dexamethasone. At low dosages of dexamethasone, after 19 days of treatment, we found no statistically significant differences as compared to the basal values. Adaptation to dexamethasone, the lower dosage and/or the improved clinical condition of the infants could be the explanations for the difference found between day 4 and day 19.

The decrease in weight gain shown in the present study during high dexamethasone has been demonstrated before [7,22], although there is also a study that does not show an effect of dexamethasone on weight gain [8]. Since energy intake was not different and ventilatory settings improved, it can be stated that the decrease in weight gain should be attributed to the high dosage therapy. The long term effect on weight is not known, but Cummings *et al.* showed that 50% of the infants who had been receiving dexamethasone had a weight < 5th percentile (n=12) at 15 months of age, whereas only 20% (n=5) of a control group with similar initial respiratory settings had a weight < 5th percentile [6].

Together with the sharp decrease in weight gain during the first week of treatment, we noticed very high urinary nitrogen excretion rates at day 4. The only other study in preterm infants on dexamethasone therapy in VLBW infants that focussed on nitrogen excretion found a reduction of 50% of the nitrogen retention at a high dosage of dexamethasone (0.6 mg.kg^{-1}.d^{-1}) [19]. Mean protein retention dropped to 20% of the initial value at day 4 in the present study. The impairment of protein accretion could result from decreased protein synthesis and/or increased proteolysis. Nitrogen balances only allow estimates of net changes in whole body protein metabolism. The use of isotope tracers enables us to measure rates of protein synthesis and breakdown in addition to protein balance. In the study presented here, we show that the rate of appearance of leucine is markedly increased at day 4 of the dexamethasone therapy in most infants. Leucine, an essential amino acid, can not be synthesized by the infant. The rate of appearance of leucine can therefore only be

Figure 7.2. Nitrogen data before (day 0) and during the dexamethasone course. Mean ± SEM. Open bars represent the intake, hatched bars show the urinary nitrogen excretion and filled bars represent the nitrogen retention. ** indicates a difference from basal values at $P \leq 0.01$, *** indicates a difference from basal level at $P \leq 0.005$.



increased by either an increased intake, or by an increased whole-body protein breakdown. Leucine intake did not change markedly on the study days, indicating that whole-body protein breakdown increased as a result of the dexamethasone treatment. This is in agreement with the data of Beaufrère *et al.*, who also showed an increase of 31% in adults [15]. Because of the higher turnover rates in preterm infants compared to the adults studied by Beaufrère *et al.*, the absolute increase of endogenous leucine appearances is almost 4 times higher in preterm infants compared to adults.

Most studies in rats and human adults show either no effect on protein synthesis or a decrease in protein synthesis following corticosteroids [10,11,13-15,18,20,32-34]. In the present study, we show that protein synthesis is not impaired during administration of corticosteroids, it even tends to be higher. The catabolic effect of dexamethasone in preterm infants is thus not caused by a decrease in synthesis, but is an effect of an increased breakdown.

In three infants at day 19 of the treatment, the tracer was administered orally.

This could have resulted in a splanchnic uptake of tracer and a lower enrichment in the systemic circulation. Erroneously high rates of appearance could be resulting from this splanchnic uptake. This issue has been an issue of recent investigations [35-38]. The oxidation of leucine in the splanchnic area is not a cause of significant loss of tracer [36]. Almost all leucine taken up by the splanchnic area is released as α-KICA in adults [35,37], but in very-low-birth weight infants there might be some loss of label [38]. In the present study, only one infant who received the tracer orally had a higher leucine flux on day 19 compared to before treatment started.

The mechanism of action of dexamethasone can not be determined from the present study. The activity of skeletal muscle branched chain α-keto acid dehydrogenase, the enzyme for branched chain amino acid catabolism, is thought to be increased during cortisone treatment [39], which is in agreement with the present study. We noticed a marked increase of leucine oxidation, on average twice as high on day 4 compared to before treatment was started. Sapir *et al.* and Muhlbacher *et al.* showed an increased nitrogen transport from the peripheral tissues to the liver *via* alanine and glutamine [19,40] and the liver has an increased uptake of amino acids [41]. The role of corticosteroids within the hepatocyte is less clear. Carbamoyl phosphate synthetase-I activity is increased, but ornithine transcarbamoylase activity is decreased in fetal rat liver following cortisone treatment [42]. Urea is however produced at a higher rate [21], again in agreement with our results. We showed a higher total urinary nitrogen excretion during treatment and the main part of urinary nitrogen consists of urea.

In the present study we show that high dosages of dexamethasone in very-low-birth weight infants diminishes weight gain and impairs the nitrogen balance. The latter is a result of an increased proteolysis and not of a decrease in protein synthesis. The protein kinetics kinetics measured on day 19 were not statistically different from the basal values. The detrimental effects should be taken into account upon decision when and if to start with dexamethasone treatment, especially for those infants who have milder degrees of BPD.

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CHAPTER 8

DISCUSSION

DISCUSSION

The most rapid weight gain of the human being takes place at a post conceptional age of 8-36 weeks. The fetus approximately doubles its weight every month during this period. When born prematurely, the infant is not viable before 22 weeks. With increasing age, the chance of survival increases rapidly, the survival rate is 90% at 32 weeks [1]. The postnatal weight gain is usually not as rapid as the weight gain in utero. Especially for the very immature infants (< 30 weeks of gestation) it takes more than three weeks to regain birth weight. Since nitrogen retention is a *conditio sine qua non* for growth, insight in nitrogen metabolism of the preterm infant is very important. Using stable isotopes it is possible to gain more insight into protein synthesis and breakdown processes. However, several assumptions are being made using this technique and many variables influence the results.

Which tracer to be used ?

The choice of a tracer is very important when measuring protein turnover rates. Branched chain amino acids, like leucine, can be oxidized in liver as well as in muscle. Phenylalanine however is exclusively hydroxylated in the liver. Different results for whole-body protein kinetics can thus be obtained with the simultaneous use of different tracers within the same patient, since different tracers might reflect different tissues that respond differently to the pertubations. However in certain conditions, different tracers can yield the same conclusion [2]. Bier has compiled flux rates of a number of amino acids and related them to their proportionate contribution to whole body protein content [3]. He found a reasonably good linear relation between the individual amino acid turnover rates and their contribution to protein composition. This is however remarkable if one realizes that the different proteins in the body do not contribute to whole-body protein turnover in proportion to their relative masses. Muscle protein for instance, although a very large pool of protein, will not contribute to a great extent to the whole-body protein turnover, because the muscle turnover rate is slow. Proteins produced in the liver, although less significant in mass, might contribute much more because of their relatively higher turnover rate. This would not make a difference if the composition of *e.g.* liver proteins would be identical to muscle proteins, but this is not the case.

The extrapolation of individual amino acid kinetics to whole-body protein kinetics enables a comparison between studies with different tracers and/or different

approaches. Both the classical approach (with the use of [^{15}N]glycine) and the representative amino acid tracer approach (with the use of [1-^{13}C]leucine) are used in this thesis. Both tracers were compared simultaneously in orally fed, stable, growing preterm infants. The study is described in chapter 3.

It was shown that the mean protein synthesis rates (AGA and SGA infants) using [1-^{13}C]leucine was 12 g.kg^{-1}.d^{-1}, whereas the mean protein synthesis rate using [^{15}N]glycine and based upon NH_3 enrichment was 11 g.kg^{-1}.d^{-1}. Whole-body protein synthesis rates in AGA infants based upon enrichment in urinary urea, was 12 g.kg^{-1}.d^{-1}. These results show that both tracers yield similar results in this group of orally fed preterm infants. However, the individual turnover rates did not correlate. In one infant a high whole-body protein turnover rate could be found using [^{15}N]glycine as a tracer, while a relatively low whole-body protein turnover rate was obtained with [1-^{13}C]leucine as a tracer. A very interesting finding was that SGA infants had very low enrichments in urinary urea following [^{15}N]glycine administration. This was also found in AGA infants devoid of amino acids on postnatal day 3 of life (chapter 2). Both findings might be attributed to the semi-essentiality of glycine, in other words, the capability of the infant to synthesize glycine falls short to meet the needs for glycine. Most tracer molecules are probably used for protein biosynthesis and the labelled nitrogen of glycine is not excreted as urea in the urine. Since the origin of urinary ammonia is completely different (kidney *vs.* liver for urea), both end-products yield different enrichments. It might not be surprising that the preterm infant devoid of glycine intake, is not able to produce adequate amounts of glycine. However, the indication that the, *in utero* growth retarded, preterm infants at 3-4 weeks of age are unable to meet the glycine needs is remarkable.

The hypothesis of the semi-essentiality of glycine is further supported by the finding of Cauderay *et al.* showing a plateau in both urinary urea and ammonia following [^{15}N]glycine administration with a 50% higher intake of glycine (90 mg.kg^{-1}.d^{-1}), compared to the intake in our study (60 mg.kg^{-1}.d^{-1}) [4]. The same research group did not find enrichment in urinary urea in half of the infants receiving a lower amount of glycine (60 mg.kg^{-1}.d^{-1}) [5].

The different enrichment of urea and ammonia following [^{15}N]glycine administration in certain circumstances implies that [^{15}N]glycine is not always suitable to calculate whole-body protein turnover rates.

The effect of early administration of amino acids

Three chapters in this thesis (2,4,6) deal with the effect on protein kinetics of starting amino acid administration on different postnatal days. Although mortality and long-term morbidity were not examined, it is shown that a beneficial effect is present on the day of commencement. In chapter 2, a study is described where two groups of infants are compared. At an energy intake of less than 50 $kcal.kg^{-1}.d^{-1}$, one group received amino acids from day 2 onwards, whereas the other group did not receive amino acids for the first four days of life. The latter group lost nitrogen, but the group that was supplemented gained 1.1 g amino acids per kilogram per day. The increased nitrogen retention resulted from a higher protein synthesis, protein breakdown was not significantly altered. The measurements were made on day 3 and 4, using [^{15}N]glycine as a tracer.

In chapter 4, a similar study is described but this study was conducted on the first day of life. Again two groups were compared, one of the groups receiving amino acids almost directly from birth onwards, the other group started after the study was finished. Supplementation of amino acids resulted in a significantly higher nitrogen and leucine retention, even at an energy intake of less than 30 $kcal.kg^{-1}.d^{-1}$. No statistical significant differences were found in synthesis and breakdown rates. with [1-^{13}C]leucine as a tracer.

A very interesting finding in the group who received amino acids from birth onwards was that the plasma level of the only amino acid that remained below the reference value (cystine) was strongly related to protein synthesis ($r = 0.83$). It supports the concept that a low availability of just one amino acid can affect protein synthesis.

There are studies describing that pertubations in amino acid metabolism exert their effect over a long time. The two groups of infants studied on the first postnatal age (described in chapter 4) were studied again on postnatal day 7. No difference in nitrogen metabolism were found between the group starting within a few hours of birth or the group starting on postnatal day 2 when measured after one week. From the studies described in this thesis, it can not be deduced whether the effect of early administration is limited to the first postnatal day or might persist over a few more days. Protein turnover studies are needed on every consecutive day from birth onwards to answer this question.

The effect of differences in composition of amino acid solutions

Not only the time of commencement of amino acid administration is important, the composition of the amino acid solution might also influence protein metabolism. Cysteine and tyrosine are amino acids considered to be essential for the preterm neonate and should therefore be supplied. The solubility and stability is however low, indicating that other manners of substitution have to be found. Acetylation of amino acids improves the stability and solubility, enabling larger amounts of those amino acids to be dissolved in the solution. In chapter 5, it was shown that a large fraction of the administered acetylated amino acids (tyrosine and cystine) was excreted in the urine, while the plasma levels of the acetylated tyrosine and cystine were higher than the levels of tyrosine and cystine itself. The poor hydrolysis might be due to the route of administration. Intestinal mucosa is the most potent hydrolysing tissue, which is mainly bypassed in the infants studied, because of the parenteral administration of the amino acids. The plasma levels of cystine were very low in infants receiving the solution containing acetyl-cysteine. This was not only due to the high urinary excretion of acetyl-cysteine, but also to the very low intake. Infants receiving a different amino acid solution containing an almost 3 times higher concentration of cysteine-HCl, had plasma levels within the reference range.

In another study, described in chapter 6, we looked at protein kinetics in infants receiving amino acid solutions with different chemical compositions. In partially different groups of infants we could not detect differences in protein kinetics. This was surprising since we had shown that plasma cystine concentration was highly related to protein synthesis rate. There was however a difference in nitrogen retention between the two groups. There are several possible explanations, why we did not find significant differences:

Firstly, the study conditions were different. On postnatal day 7, both groups of infants received $\pm$ 2 g amino acids.$kg^{-1}.d^{-1}$ and $\pm$ 70 kcal.$kg^{-1}.d^{-1}$, whereas the infants on day 1 received $\pm$ 1 g of amino acids and $\pm$ 30 kcal.$kg^{-1}.d^{-1}$. Secondly, whereas plasma cystine concentration was low in infants receiving the solution containing acetylated cystine, plasma tyrosine concentration was low in infants receiving the other amino acid solution. A low level of tyrosine might affect protein kinetics as well, thereby minimizing the differences between the two groups. Thirdly, the method of tracing whole-body protein kinetics used might not be sensitive enough to detect small differences, or the tracer used might not be reflecting protein turnover in the tissue(s) where the largest changes take place.

Finally, protein synthesis rates could already be near maximal rate at this energy and amino acid intake.

Hormones and nitrogen metabolism

The nutritional effects on nitrogen metabolism as discussed in chapters 2,4 and 6 are probably partly mediated indirectly *via* hormones and partly mediated directly. Leucine, for instance, stimulates protein synthesis directly. However, administration of amino acids usually lowers glucose levels, which is attributed to a rise in insulin concentration [6]. Insulin itself is found to decrease protein breakdown in human adults, which is the indirect effect.

Most infants described in this thesis, were preterm infants within the first week of life. The amount of stress caused by the clinical condition, handling procedures (and by the delivery itself in the infants studied on the first day of life) increases protein turnover rate *via* hormonal actions.

The most obvious example of the influence of clinical condition on protein kinetics is shown by the protein turnover rates found in the infants studied on the first day of life. Protein turnover and breakdown rates were very high, despite very low energy and amino acid intake. Cortisol and catecholamines are known to be high during the first day of life, and they are both known to have a stimulatory effect on protein turnover rates.

The effect of glucocorticoids on protein metabolism in preterm infants with bronchopulmonary dysplasia is presented in chapter 7. Following high dosages of dexamethasone, both whole-body protein turnover and breakdown rates increased. Protein synthesis was not significantly altered. In trying to reduce the catabolic effect of dexamethasone, one should mainly focuss on lowering protein breakdown rates. This might be achieved *via* insulin administration, because insulin stimulates nitrogen retention particularly through lowering protein breakdown rates. Growth hormone infusion also resulted in enhanced nitrogen retention in human adults receiving glucocorticoids, but through an increase in protein synthesis [7,8]. Glucose metabolism should be carefully monitored in such studies.

The study of the effect of dexamethasone shows how stable isotope studies might contribute to the understanding of pertubations in protein kinetics and give directions to future therapy and research.

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CHAPTER 9

SUMMARY/SAMENVATTING

SUMMARY

Growth is often impaired in very-low-birth weight infants born premature. Since protein accretion is very important for growth, it is not surprising that there is a general interest to learn more about protein metabolism in infants who should be in their most rapid growth period of their life. This thesis describes the nitrogen metabolism of preterm infants in different conditions. Most of the studies are performed with infants in their first week of life, whereas two studies are performed with infants around one month of age.

Chapter 1 gives an introduction on the techniques used and summarizes the hormonal regulation of protein metabolism. The available literature on protein metabolism in premature infants is cited.

Chapter 2 describes a study comparing the administration of amino acids starting on postnatal day 2 *versus* day 4. The effects on nitrogen metabolism are measured using [^{15}N]glycine as a tracer and by performing 48 hr nitrogen balance. The administration of amino acids from day 2 onwards resulted in a higher nitrogen accretion due to an improved protein synthesis.

In chapter 3, two commonly used tracers, [^{15}N]glycine and [1-^{13}C]leucine are compared in 4 week old, growing, preterm infants receiving oral feeding. Group means using both tracers were very comparable, but the individual values did not correspond. Following [^{15}N]glycine administration urea did not become significantly enriched in infants with intra-uterine growth retardation, implying that this method is not always suitable to measure protein kinetics in preterm infants.

Chapter 4 describes the effect on nitrogen metabolism of starting amino acid administration within a few hours of birth. Plasma amino acid levels normalized following amino acid administration. Nitrogen retention improved, although protein synthesis and breakdown rates were not significantly altered. The plasma level of cystine, the only amino acid below the reference range in the group of infants receiving amino acids, was highly predictive for protein synthesis.

The effect of administering acetylated tyrosine and cysteine to preterm infants is described in chapter 5. A large part of these acetylated amino acids were excreted in the urine, wheras the deacetylation was not very active. The acetylated tyrosine and cystine levels were higher than the non-acetylated levels in plasma.

Chapter 6 describes a comparative study between three groups of preterm infants, receiving different amino acid solutions and starting either from birth onwards or on postnatal day 2. The infants were studied on day 7 of life. No

differences were found in nitrogen metabolism between infants starting from birth onwards and infants starting at day 2. Infants receiving acetylated tyrosine and cysteine had lower nitrogen retention compared to infants receiving an amino acid solution without acetylated amino acids.

Chapter 7 describes the effect of dexamethasone therapy to preterm infants with lung disease (bronchopulmonary dysplasia) on nitrogen metabolism. It was demonstrated that high dosages of dexamethasone increased protein metabolism resulting in a lower nitrogen retention. After 19 days of treatment and at a lower dosage of dexamethasone, no significant effects were noticed on protein metabolism.

In this thesis it is shown that the early commencement of amino acids improves nitrogen balance, even at a very low energy intake. The effect of early administration seems limited to the first few postnatal days. The chemical compostion of the amino acid solution is important with regard to amino acid profiles and nitrogen retention. Acetylation of some amino acids enables administration of sufficient unstable or unsoluble essential amino acids, but the parenterally fed preterm infant does not seem to utilize these acetylated amino acids very well. Other manners have to be found to supply parenterally fed preterm infants with essential amino acids that are unstable or less soluble.

Many tracers can be used to monitor changes in protein kinetics. [^{15}N]glycine is not a suitable tracer to study whole-body protein kinetics in preterm infants under the circumstances the infants were studied in this thesis. With [1-^{13}C]leucine as a tracer it was shown that a very commonly used therapy for infants with chronic lung disease (tapered dexamethasone course) resulted in increased protein breakdown.

SAMENVATTING

Vergeleken met de intra-uterine gewichtstoename is de groei bij premature met een zeer laag geboortegewicht vaak lager. Een belangrijke parameter voor groei is de opslag van eiwit. Het is daardoor niet verwonderlijk dat er veel interesse bestaat voor het eiwitmetabolisme van kinderen die zich in een fase bevinden waarin de snelste groei van hun leven plaatsvindt.

In hoofdstuk 1 wordt een inleiding gegeven waarin de achtergronden van de studies worden beschreven. Tevens worden de technieken die gebruikt zijn besproken alsmede wat er bekend is over het eiwitmetabolisme in de literatuur.

In hoofdstuk 2 wordt een studie beschreven waarbij twee groepen kinderen vergeleken. Eén groep kinderen ontving vanaf de 2e levensdag aminozuren, terwijl de andere groep op de 4e levensdag startte. Het eiwitmetabolisme werd bestudeerd met behulp van [^{15}N]glycine als tracer en door het uitvoeren van een 48 uur durende stikstofbalans. Het vroege toedienen van aminozuren resulteerde in een verbeterde stikstofretentie als gevolg van een verhoogde eiwitopbouw.

In hoofdstuk 3 worden twee veel gebruikte tracers vergeleken, [^{15}N]glycine en [1-^{13}C]leucine bij 4 weken oude, groeiende prematuren die oraal werden gevoed. De gemiddelde eiwit synthese en afbraak kwam, gemeten met beide tracers, goed overeen. De individuele getallen kwamen echter niet goed overeen. Het ureum van dysmature kinderen werd nauwelijks verrijkt na toediening van [^{15}N]glycine, waardoor deze tracer niet altijd geschikt lijkt voor het meten van eiwitmetabolisme in deze groep kinderen.

In navolging van het onderzoek beschreven in hoofdstuk 2, werd het effect vergeleken van het geven van aminozuren vanaf de geboorte en vanaf dag 2. Hoofdstuk 4 beschrijft dit onderzoek, waaruit bleek dat de plasma aminozuur spiegels normaliseerden na het geven van aminozuren en dat de stikstof retentie verbeterde. De eiwit synthese en afbraak veranderden echter niet significant.

Het effect van het geven van geacetyleerde aminozuren wordt beschreven in hoofdstuk 5. Een groot deel hiervan wordt uitgescheiden in de urine. De de-acetylatie is niet erg actief bij premature kinderen in de eerste levensweek. Het gevolg was dat de geacetyleerde spiegels van tyrosine en cystine hoger waren dan die van de niet geacetyleerde tyrosine en cystine.

In hoofdstuk 6 wordt een studie beschreven waarbij drie groepen prematuren worden vergeleken. Eén groep ontving aminozuren vanaf de geboorte, één groep vanaf dag 2 en één groep ontving vanaf dag 2 een aminozuur oplossing met enkele

geacetyleerde aminozuren. Het eiwitmetabolisme bij deze kinderen werd gemeten op dag 7. Geen verschil werd meer gezien tussen de kinderen die vanaf de geboorte aminozuren kregen toegediend en de kinderen die dit vanaf dag 2 kregen. De kinderen die de geacetyleerde aminozuur oplossing kregen hadden een significante lagere stikstof retentie in vergelijking met de kinderen die een aminozuur oplossing zonder geacetyleerde aminozuren toegediend kregen.

Het effect van het geven van dexamethason als therapie voor bronchopulmonaire dysplasie op het stikstof metabolisme wordt beschreven in hoofdstuk 7. Hoge dosis dexamethason resulteerde in een verhoogde eiwitafbraak, terwijl de eiwitsynthese niet statistisch significant veranderde. Na 19 dagen van de dexamethason kuur, met een lagere dosis, was het effect niet meer meetbaar.

Uit dit proefschrift blijkt dat het vroeg starten met de toediening van aminozuren een gunstig effect heeft op de stikstofbalans, ondanks de geringe hoeveelheid toegediende energie. De chemische samenstelling van de aminozuur oplossing heeft een duidelijk effect op de plasma aminozuur spiegel terwijl ook de stikstofbalans erdoor beinvloed lijkt. Het acetyleren van moeilijk oplosbare essentiële aminozuren maakt het toedienen van voldoende hoeveelheden mogelijk maar pasgeboren prematuren lijken geacetyleerde aminozuren niet goed te gebruiken. Andere manieren dienen te worden gevonden om moeilijk oplosbare en instabiele essentiële aminozuren aan pasgeboren te geven.

Vele tracers kunnen worden gebruikt om eiwitmetabolisme te bestuderen. [^{15}N]glycine lijkt bij premature pasgeborenen niet de tracer van eerste keus, terwijl [1-^{13}C]leucine wel geschikt lijkt. Met behulp van deze laatste tracer is gebleken dat dexamethason therapie (een veel gebruikte therapie bij preterme kinderen met een chronische longaandoening) tot een verhoging van de eiwitafbraak leidt. Deze therapie wordt zeer veel gebruikt bij kinderen met een chronische longaandoening.

SAMENVATTING VOOR NIET-MEDICI

Tijdens de zwangerschap groeit het ongeboren kind erg snel, sneller dan het kind ooit nog zal doen. De gewichtstoename gedurende bijvoorbeeld de 4e tot de 8e maand bedraagt meer dan 15 gram per kilo per dag. Voor een volwassene houdt een vergelijkbare gewichtstoename in dat iemand van 70 kilo binnen een maand meer dan 100 kilo weegt.

Deze gewichtstoename blijkt haalbaar wanneer een kind te vroeg geboren wordt. Kinderen met een zeer laag geboortegewicht doen er vaak 2 tot 4 weken over voordat het geboortegewicht weer bereikt is. Bij ernstig zieke kinderen is het ook daarna vaak moeilijk om ze net zo snel te laten groeien als in de baarmoeder.

Een zeer belangrijk onderdeel van de groei is het vasthouden en gebruiken van eiwit. Uit vele studies is gebleken dat het eiwit gehalte van nieuw gevormd weefsel altijd voor 10 tot 15% uit eiwit bestaat. Zonder eiwit is men niet in staat te groeien.

Eenmaal opgebouwd eiwit ligt niet voor jaren vast. Reeds in 1939 is ontdekt dat de eiwitten in ons lichaam continu worden afgebroken en weer gesynthetiseerd. Eiwitten bestaan uit kleinere delen: aminozuren, waarvan er ruim 20 zijn. Door de volgorde, soort en hoeveelheid aminozuren te wisselen maakt het menselijk lichaam duizenden verschillende eiwitten. De aminozuren bestaan weer uit atomen, waarvan, en dat is het kenmerk van eiwit, er altijd één een stikstof atoom is. Door één van deze atomen te vervangen door een zelfde soort, maar iets zwaarder atoom (stabiel isotoop) krijgt men een gemerkt aminozuur (tracer), waarmee het mogelijk is de eiwit opbouw en afbraak te meten. Vroeger werd er voor dit soort onderzoek veel gebruikt gemaakt van radio-actief gemerkte aminozuren. Toepassing hiervan is niet mogelijk bij kinderen vanwege het nadelige effect van radio-actieve straling op groeiende cellen. In dit proefschrift staat veel onderzoek beschreven waarbij gebruik is gemaakt van aminozuren die voorzien waren van stabiele isotopen.

In hoofdstuk 1 wordt een overzicht gegeven van de technieken die gebruikt zijn en wat er bekend is in de internationale literatuur met betrekking tot voeding en eiwit stofwisseling bij te vroeg geboren kinderen. Onder verschillende omstandigheden zijn ± 150 te vroeg geboren kinderen onderzocht die hun voeding door een slangetje via de neus in de maag kregen toegediend. Daarnaast zijn van ruim 80, via een infuus gevoede, kinderen de gegevens betreffende het eiwit metabolisme bekend. In dit proefschrift worden daar de gegevens van bijna 100 kinderen aan toegevoegd.

Vrijwel overal op de wereld krijgen te vroeg geboren kinderen alleen wat suiker via een infuus gedurende de eerste paar dagen. Op zich is dat verbazingwekkend omdat kinderen in de baarmoeder via de moederkoek en navelstreng wel eiwit krijgen en het bekend is dat kinderen zonder eiwit niet groeien. De reden om te vroeg geborenen geen eiwit te geven is dat er in het verleden veel bijwerkingen ontstonden bij het geven van de eiwit oplossing. De samenstelling van deze eiwit oplossingen was niet optimaal. Sinds kort zijn deze oplossingen echter aanzienlijk verbeterd. Zowel in hoofdstuk 2 en 4 beschrijf ik wat het effect is van het geven van eiwit gedurende die eerste levensdagen. In hoofdstuk 2 vergelijk ik twee groepen op de derde en vierde levensdag. Eén groep krijgt eiwit, de andere niet. Beide groepen zijn verder vergelijkbaar met betrekking tot de totale hoeveelheid calorieën die ze krijgen, het geboorte gewicht en de mate van ziek zijn. Uit deze studie blijkt dat de kinderen die eiwit krijgen daar ook een belangrijk deel van gebruiken om lichaamseiwitten zoals spieren op te bouwen. De groep die geen eiwit krijgen breken daarentegen meer eiwit af dan ze opbouwen en verliezen dus netto lichaamseiwit.

In hoofdstuk 4 wordt het onderzoek herhaalt, maar de meting vindt nu plaats op de eerste levensdag. Weer worden twee groepen kinderen vergeleken, één groep met eiwit en suiker als voeding vanaf de geboorte, de andere groep met alleen suiker als voeding. Wederom is er een verschil in de hoeveelheid eiwit die vastgehouden / verloren wordt. Het is opmerkelijk dat de kinderen zo'n verschil in het vasthouden van eiwit lieten zien omdat ze slechts zeer weinig calorieën toegediend kregen. Algemeen werd aangenomen dat er een bepaalde minimale hoeveelheid energie moest worden gegeven om de kinderen ook het gegeven eiwit te laten gebruiken. Uit de studie blijkt nu dat het vasthouden van eiwit al plaats vindt een marginale energie inname. Een deel van hoofdstuk 6 beschrijft een volgende studie waarin bekeken wordt of er nog een verschil in eiwit stofwisseling bestaat na een week tussen kinderen die vanaf de geboorte eiwit krijgen en kinderen die vanaf de 2^{e} levensdag eiwit krijgen. Er is op de leeftijd van 7 dagen echter geen verschil meer aan te tonen tussen beide groepen, zodat het lijkt alsof er geen langdurig aantasting van de eiwit opbouw plaats vindt door een onderbreking van de eiwit toevoer in de eerste paar levensdagen.

Zoals hierboven beschreven is maakt men gebruik van gemerkte aminozuren (tracers) om het proces van eiwit opbouw en afbraak te bestuderen. In principe kunnen alle (± 20) aminozuren gebruikt worden om de eiwit stofwisseling te meten. In hoofdstuk 3 beschrijf ik een onderzoek waarbij de twee meest gebruikte vergelijk ([^{15}N]glycine en [1-^{13}C]leucine). Niet alleen het aminozuur zelf is verschillend, ook

het deel wat gemerkt is (stikstof (Nitrogen) of koolstof (Carbon)) verschilt. Nog nooit waren deze twee tracers tegelijkertijd bij te vroeg geborenen vergeleken. Het blijkt dat de groepsgemiddelden voor eiwit opbouw en eiwit afbraak goed overeenkomen gemeten met beide tracers. Echter de individuele getallen correleerden niet. Het model om de eiwit stofwisseling te meten voldeed niet bij één van de tracers ([^{15}N]glycine), een fenomeen wat ook al optrad in de studie beschreven in hoofdstuk 2. De conclusie luidde dan ook dat [^{15}N]glycine niet geschikt om bij deze groep kinderen, onder deze omstandigheden, de eiwit stofwisseling te meten. Alle studies zijn daarna dan ook uitgevoerd met [1-^{13}C]leucine als tracer.

Niet alleen het tijdstip van het starten met eiwit toediening is van belang, ook de kwaliteit van de eiwit oplossing bepaalt of te vroeg geboren kinderen het eiwit kunnen gebruiken. In hoofdstuk 5 vergelijk ik drie, momenteel verkrijgbare aminozuur oplossingen met een verschillende chemische samenstelling. Het verschil ligt voornamelijk in het voorkomen van geacetyleerde aminozuren. Dit zijn stukjes eiwit die, omdat ze slecht oplosbaar of onstabiel zijn, voorzien zijn van een extra deeltje wat hen stabieler en beter oplosbaar maakt. Echter om deze geacetyleerde aminozuren te kunnen gebruiken moet het kind dat extra stukje eraf halen (de-acetyleren). Het blijkt dat te vroeg geboren kinderen die via een infuus worden gevoed dat niet goed kunnen. Het gevolg is dat de spiegels van de geacetyleerde aminozuren veel hoger zijn dan de spiegels van de gede-acetyleerde aminozuren. Verder wordt ook ongeveer 50 % van de gegeven hoeveelheid geacetyleerde aminozuren in de urine uitgescheiden.

Het effect van de verschillende chemische samenstelling op de eiwit stofwisseling wordt beschreven in hoofdstuk 6. Het lijkt dat kinderen die eiwit deeltjes met dat extra stukje eraan toegediend krijgen minder goed gebruik kunnen maken van het eiwit. Wanneer twee groepen worden vergeleken die evenveel eiwit krijgen, blijkt de groep die geacetyleerde aminozuren toegediend krijgt minder eiwit te kunnen vasthouden.

Doordat kinderen te vroeg geboren worden zijn sommige organen, zoals de longen, nog niet volledig tot ontwikkeling gekomen. Deze kinderen hebben dan ook soms extra zuurstof en beademingsapparatuur nodig om het zuurstof gehalte in het bloed op een voldoende hoog niveau te houden. Zonder voldoende zuurstof treedt er bijvoorbeeld in de hersenen beschadiging op. Door de beademing en de toediening van zuurstof treedt er echter soms long beschadiging op, waardoor weer zeer langdurig behandeling met beademing noodzakelijk is. Met behulp van een kuur met

een synthetisch hormoon (dexamethason) is het soms mogelijk van deze ernstige ziekte te genezen. In hoofdstuk 7 wordt de invloed van dit hormoon op de eiwit stofwisseling en groei beschreven. Met behulp van [1-^{13}C]leucine als tracer werd op drie tijdstippen de eiwit stofwisseling bestudeerd. Metingen vonden plaats voorafgaande aan de kuur, tijdens een hoge dosering dexamethason en tijdens een lage dosering. Het blijkt dat een hoge dosering van het medicijn de eiwit afbraak duidelijk verhoogde, terwijl de eiwit opbouw niet significant veranderde. Tijdens de lage dosering van dexamethason was er geen effect op de eiwit stofwisseling. Hetzelfde patroon was aanwezig met betrekking tot de groei. Op de hoge dosering in de eerste week van de behandeling bleven de kinderen vrijwel constant in gewicht, sommige vielen zelfs af, in plaats van met $\pm$ 10% in gewicht toe te nemen. Op de lage dosering was de gewichtstoename weer goed, zelfs iets beter in vergelijking met de groei snelheid in de baarmoeder (de gouden standaard).

Door middel van deze studie is het nu bekend dat, om de bijwerking van dit medicijn teniet te doen, de stijging van de eiwit afbraak moet worden tegengegaan. Dit vergt een andere behandeling dan welke zou moeten plaatsvinden wanneer de eiwit opbouw ernstig verstoord zou zijn. Hiermee is een belangrijke stap gezet om te vroeg geboren kinderen die vaak al problemen hebben met het groeien, zo min mogelijk nadelig effect van hun behandeling te laten hebben.

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CURRICULUM VITAE

1962	Geboren te Laren (NH) op 7 december
1975-1981	Ongedeeld VWO, Het Nieuwe Lyceum te Hilversum
1981-1982	Propadeuse geneeskunde Erasmus Universiteit Rotterdam
1982-1984	Kandidaats geneeskunde
1984	Keuze-onderzoek: Intracellulaire overdracht van genen onder invloed van ioniserende straling. (o.l.v. Prof.Dr. D.W. van Bekkum, TNO Rijswijk)
1985-1987	Doctoraal geneeskunde
1985-1987	Medisch studenten team psychiatrie. Academisch Ziekenhuis Rotterdam-Dijkzigt.
1987	Onderzoek: Uitscheiding van organische en anorganische zuren van patiënten met het androgeen ongevoeligheids syndroom. (o.l.v. Prof.Dr. H.J. Degenhart, Sophia Kinderziekenhuis Rotterdam)
1987-1989	Co-schappen, artsexamen
1987-1988	Medisch nachthoofd. Residence Essenburgh, Rotterdam.
1988-1989	Keuze co-schappen kinderendocrinologie (Karolinska Institutet, Stockholm Zweden) en kindercardiologie (St Görans Sjukhuset, Stockholm, Zweden) Onderzoek: Effect van groeihormoon op de hartspier. (o.l.v. Prof. M Ritzen, Karolinska Institutet, Stockholm, Zweden)
1989-1993	Onderzoeker in opleiding (NWO). Promotie-onderzoek (Sophia kinderziekenhuis Rotterdam, afdeling Pasgeborenen, promotor Prof.Dr. P.J.J. Sauer)
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