Urea and Non–Protein Nitrogen Metabolism in Infants

With Special Reference to the Sudden Infant Death Syndrome (SIDS)

BY

MARY GEORGE
ABSTRACT


A large amount of non – protein nitrogen, in the form of urea and ammonium, is present in human breastmilk; however its physiological role in the infant is as yet not fully understood. It has been hypothesized that an insufficient enteric metabolism of urea could play a role in the sudden infant death syndrome (SIDS). This thesis was undertaken to study the enteric metabolism of non – protein nitrogen in healthy infants, in comparison with those who had succumbed to SIDS.

Aerobic and anaerobic faecal microflora, were studied in healthy infants from birth to 6 months of age. They were found to appear in faeces within 3 days of birth and were present throughout the first 6 months of life. The effect of nitrate, nitrite and nitric oxide on faecal urease activity was investigated and found to be inhibitory in action. The sigmoid faecal urease activity and sigmoid faecal urea content of SIDS infants were compared to those of control infants; significantly lower sigmoid faecal urease activity and greater sigmoid faecal urea content were found in the SIDS infants. The total number of SIDS cases occurring in Sweden during the period 1990 through 1996 was analysed regarding geographical and seasonal distribution, in relation to the nitrate concentration in drinking water and changes in the groundwater level. The northernmost parts of the country had its highest incidence when the rest of the country had its lowest incidence, and the occurrence of individual deaths was associated with the recharge of groundwater, which is known to increase its nitrate content. The effect of ingested ammonium on carbon dioxide production was determined in healthy infants using the doubly labelled water technique. No change in carbon dioxide production was observed.

An insufficient enteric metabolism of urea in infants and peak or greatly varying nitrate concentrations in drinking water are associated with the occurrence of SIDS. Ingested ammonium supplements in the given doses did not influence carbon dioxide production in healthy infants.

Key words: Urea, non – protein nitrogen metabolism, SIDS, drinking water nitrate concentration, carbon dioxide production.

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“There is no finer investment for any community than putting milk into babies.”

Winston Churchill  1943
This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


IV. The effect of ammonium chloride ingestion on carbon dioxide production in infants determined by using the doubly labelled water technique. George M, Forsum E, Wright A, Coward WA, Wiklund L. Submitted for publication.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^2$H</td>
<td>Stable isotope, deuterium</td>
</tr>
<tr>
<td>$^{18}$O</td>
<td>Stable isotope</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DLW</td>
<td>Doubly labelled water</td>
</tr>
<tr>
<td>H$^+$</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>Bicarbonate ion</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Dihydronicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>Ammonium ion</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>SIDS</td>
<td>Sudden infant death syndrome</td>
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</table>
INTRODUCTION

As early as 1949 it was known that human breastmilk contains large amounts of non-protein nitrogen in the form of urea and ammonium. (1) Non-protein nitrogen, half of which is made up of urea nitrogen, accounts for 25 per cent of the total nitrogen in breastmilk. (2,3,4) The physiological role of this urea and ammonium and its contribution to normal metabolism in the infant is as yet not fully understood. In the adult, urea is considered to be a water soluble relatively nontoxic waste product, which is excreted as urine through the kidneys.

Ingested urea, contained in breastmilk, is metabolized by urease-containing bacteria, which are found in the intestines within 5 days of birth. (5,6,7,8) Currently available infant formula feeds, which are similar to breastmilk in their high lactose content and low protein content, were found to stimulate growth of intestinal Bifidobacteria but unable to suppress pathogenic bacteria such as Clostridia and Klebsiella. (9) In adults, urea produced in the liver enters the enterohepatic circulation and diffuses into the intestine, where about 20 per cent of the urea is hydrolyzed by intestinal microflora into ammonia while the rest of it is excreted unchanged in the urine. (10) The therapeutic use of antibiotics such as neomycin to abolish intestinal bacterial action on urea in patients with impending hepatic coma is well established.

In the infant, the ammonia from dietary urea is transported to the liver where it is metabolized once again to urea and excreted through the kidneys. Some of the nitrogen from dietary urea is incorporated into body proteins. (11, 12, 13)
Urea Metabolism and Acid - Base Balance

Traditional view

Ingestion of protein results in the release of amino acids into the splanchnic circulation. The deamination of amino acids, which is the removal of the amino groups from the amino acids occurs mainly in the liver. The ammonium ion together with bicarbonate is synthesized into urea via the urea cycle. Urea is then excreted through the kidneys in the urine. (14)

In the proximal tubular cells of the kidneys, deamination of glutamine yields 2 ammonium ions and 1 $\alpha$-ketoglutarate ion; the ketoglutarate ion is metabolized to glucose, or to carbon dioxide and water producing 2 bicarbonate ions. Thus a bicarbonate ion is added to the blood for each hydrogen ion that is excreted in the form of an ammonium ion. In the Loops of Henle and in the medullary interstitium the concentration of the ammonium ion increases by means of a countercurrent multiplication before being secreted into the collecting ducts. (15)

Alternative view according to Atkinson and Camien

Catabolism of protein produces a large amount of bicarbonate ion (base) rather than acid as is generally believed. Bicarbonate ions are produced from the $\alpha$-carboxyl group of an amino acid and from the carboxyl or carboxamide group of glutamate, aspartate, glutamine and asparagine as a result of amino acid metabolism. Approximately 1 mol of bicarbonate ion is produced from a daily consumption of 100 g of protein. Bicarbonate ions combine with ammonium ions synthesizing urea. Synthesis of urea supplies protons, which titrate the large amount of bicarbonate produced, thus maintaining acid - base balance. (16)

$$2\text{NH}_4^+ + 2\text{HCO}_3^- \rightarrow \text{NH}_2 - \text{CO} - \text{NH}_2 + \text{CO}_2 + 3\text{H}_2\text{O}$$
Sudden Infant Death Syndrome (SIDS)

SIDS is defined as the sudden death of an infant or young child, which is unexpected by history, and in whom a thorough post-mortem examination fails to demonstrate an adequate cause of death.

Sudden and unexpected deaths in infancy have been described to occur as early as 500 B.C. (17) Up until the early nineteenth century, overlaying of infants by adults was considered to be the cause of such infant deaths. The mother or wetnurse sharing the infant’s bed was forbidden; if death occurred, it was punishable by law in the middle ages, both in Sweden and in other European countries.

In the nineteenth century, several theories were proposed and subsequently discarded. Such was the fate of the theories known as the status thymico-lymphaticus (large thymus), hypogammaglobulinemia, overwhelming viremia, hypoparathyroidism, unstable myocardial conduction systems and cow’s milk hypersensitivity. (18)

A significant step forward was the proposition of the apnoea hypothesis in the 1970’s, (19, 20) and this led to the widespread use of apnoea monitors. Naeye, a pathologist, found certain characteristic tissue markers for hypoxia and hypoxemia in SIDS cases, thus supporting the apnoea hypothesis. (21) Histological studies of the central nervous system were conducted in the 1980’s, and a defect in the brain stem was suggested as the cause of the respiratory failure of SIDS. However, no etiological factor was found. (22)

The findings of a major epidemiological study of SIDS was published in 1988. (23) The significant maternal factors were found to be cigarette smoking during pregnancy, single civil status, age less than 20 years at first pregnancy, anaemia, use of illegal drugs and low weight gain during pregnancy; the factors in the infants were found to be low birth weight, frequent illnesses and prolonged post-natal stay in the nursery.
Adoption of the supine sleeping position instead of the prone position, as well as propaganda encouraging breastfeeding in the 1990’s, led to a pronounced drop in the incidence of SIDS in several countries worldwide. (24, 25, 26, 27)

The etiological factor or factors of SIDS have yet to be elucidated.

**Hypothesis Proposed by Wiklund**

Large amounts of non-protein nitrogen in the form of urea and ammonium ions are found in human breastmilk. (1, 4) *Bifidobacteria* and other anaerobic bacteria, which are found in the gut of both breastfed and formula-fed infants (9, 28) produce urease (6,7) which metabolizes ingested urea to ammonium and bicarbonate ions. The ammonium ions are absorbed and incorporated into protein in the infant (11, 12, 29, 30, 31, 32) or metabolized into urea. (30)

The synthesis of urea is accompanied by the production of hydrogen ions which combine with endogenously produced bicarbonate ions resulting in the formation of carbon dioxide and water. (33)

**Normal Infants**

\[
\begin{align*}
\text{Gut} & \quad \text{Urea (ingested)} \quad \text{Bifidobacteria (gut)} \quad \text{urease producing} \quad \text{NH}_4^+ \\
\text{Liver} & \quad \text{NH}_4^+ \text{ (from gut)} \quad \text{Urea} + \text{H}^+ \\
\text{Blood} & \quad \text{H}^+ + \text{HCO}_3^- \quad \text{CO}_2 + \text{H}_2\text{O} \\
\end{align*}
\]

---

*Figure 1. Metabolism in normal infants*
According to the hypothesis proposed by Wiklund in 1996 (34) a derangement of the above metabolism could lead to progressive alkalosis and eventually death as seen in SIDS infants.

**SIDS Infants**

Deficient bacterial breakdown of urea (gut) → Decreased production of NH$_4^-$ ions

↑

Decreased production of H$^+$ ions (liver)

↑

Accumulation of HCO$_3^-$ ions
Metabolic alkalosis
Compensatory hypoventilation
Accumulation of CO$_2$
Carbon dioxide narcosis
Hypoxia and death

*Figure 2. Hypothetical mechanism in SIDS*

To verify this hypothesis the following research was conducted, and the studies undertaken form the basis of this thesis.
AIMS OF THE THESIS

To elucidate the role of the urea and nitrogen metabolism in healthy infants and a possible causal role of a deficient metabolism in SIDS infants.

The specific aims of the studies were:

1. To study the faecal microflora and urease activity in healthy infants during the first six months of life.

2. To compare the faecal microflora, urea content and urease activity in SIDS infants with those of infants who died unexpectedly of other causes.

3. To determine the incidence and geographical distribution of SIDS in relation to the nitrate content of drinking water and groundwater levels.

4. To study the effect of ingested ammonium chloride on carbon dioxide production and total energy expenditure in healthy infants using the doubly labelled water technique.
SUBJECTS AND METHODS

Subjects

**Paper I.** Thirteen fullterm healthy infants were followed up from birth until 6 months of age. Faecal specimens, which were collected during the first 3 days following birth, at 2 months, at 3 months and at 6 months, were examined bacteriologically, and biochemically for urease activity and urea content.

**Paper II.** Thirty consecutive cases of unexpected infant death autopsied forensically were studied. Twenty-two of these infants were found to have succumbed to SIDS and the remaining 8 to other causes. Their faecal specimens were examined for bacteriological microflora, urease activity and urea content and compared.

**Paper III.** The total number of SIDS deaths (636 cases) occurring in Sweden during the period 1990 - 1996 was analysed regarding their geographical and seasonal distribution, in relation to the nitrate content of drinking water and changes in groundwater levels.

**Paper IV.** In 8 fullterm healthy infants, carbon dioxide production and total energy expenditure were determined, using the doubly labelled water (DLW) technique, following ingestion of ammonium supplements, and compared to when no additional ammonium was ingested. Urine pH, urea and ammonium concentrations were also analysed and compared.

Method

**Paper I and II**

**Analyses of Faecal Specimens**

Faecal specimens were collected after spontaneous defecation (Paper I), and from the sigmoid colon (Paper II).

**Bacteriological Analyses:** Small quantities of faeces from each infant were cultured aerobically and anaerobically and then analysed for the presence of microflora. In addition, in Paper I, the urease activity of each bacterial species was determined after incubation of the
specific bacterial species for 2 h in the presence of urea (0.3mol/L) and a bacteriological substrate at pH 6.8 - 6.9.

**Biochemical Analyses:** *Urea and ammonium concentrations, urease activity and gaseous concentration of nitric oxide (NO)*

Four faecal specimens each weighing approximately 40 mg were harvested from each individual and suspended in 4mL of sterile physiological NaCl solution in sterile test tubes. Two of the suspensions were preincubated anaerobically and the other two aerobically for 36h at 33°C, followed by centrifugation to separate faecal material from a clear supernatant. Before centrifugation the gas in the aerobic tubes was transferred into a syringe for immediate analysis of NO. The anaerobic preincubation was carried out in an anaerobic tight chamber containing a gas mixture of 90% CO₂ and 10% H₂ circulated over a palladium catalyst (GasPak Plus, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) while aerobic preincubation was done in ambient air sealed test tubes. (Figure 3)

![Figure 3. Preanalytical procedure](image-url)

After testing the pH of the supernatants (2 from anaerobic conditions and 2 from aerobic conditions), they were subdivided into 400 µL (600 µL in Paper II) samples (in duplicate), each of which was mixed with 3mL of sterile physiological NaCl solution forming an incubation mixture. Hence 8 incubation mixtures (4 from anaerobic conditions and another 4
from aerobic conditions) were supplemented with urea to a final concentration of 1.95±0.12
(1.91±0.4 in Paper II) mmol/L, and 8 additional incubation mixtures to a final urea
ccentration of 3.40±0.22 (3.38±0.47 in Paper II) mmol/L for subsequent incubations. Four
other incubation mixtures (2 from anaerobic conditions and 2 from aerobic conditions) were
incubated without any additional urea in order to determine the endogenous faecal content of
urea. (Figure 4) Finally to 4 test tubes containing sterile, physiological NaCl solutions, urea
was added to obtain final concentrations as stated above (2 of each concentration) and they
were also incubated in the same way (blanks). Each individual generated a total of 24 tubes
which were incubated for 30h at 33°C before analysis.

Urease activity

400µL supernatant (+3mL NaCl) → 2 aerobic samples + urea → urea 1.91±0.4 mmol/L

→ 2 aerobic samples + urea → urea 3.38±0.47 mmol/L

→ 2 anaerobic samples + urea → urea 1.91±0.4 mmol/L

→ 2 anaerobic samples + urea → urea 3.38±0.47 mmol/L

Urea content

400µL supernatant + 3mL NaCl → 2 aerobic samples

→ 2 anaerobic samples

Figure 4. Incubation mixtures

Nitric oxide (NO) concentration

The NO concentration of the gaseous atmosphere of the aerobic tubes was determined in a
chemiluminescence NO – NO₂ – NOₓ analyser (Model 42, Thermo Environmental Instuments
Inc, USA) and expressed in parts per billion (ppb).

All incubation mixtures were analysed regarding their contents of urea and ammonium ions in
a Hitachi 911 analytical system, each value being the mean of 4 analyses. The ammonium
ions thus formed were reacted in a reductive amination of $\alpha$ - ketoglutarate catalysed by glutamate dehydrogenase with concomitant oxidation of NADH, which was followed spectrophotometrically at 340 nm. Hence stoichiometric relationships existed between the amounts of NADH converted and ammonium ions formed (as a function of the actual urea concentration) in the urease reaction. Ammonium ions in the samples were assayed similarly but without urease. (Figure 5) The urea and ammonium ion concentration were expressed in mmols and $\mu$mols per gram (wet weight) faeces respectively, after blank corrections. All chemicals were of pro analysi grade from Boehringer - Mannheim, Germany.

$$\text{urease} \quad \text{Urea} \rightarrow \quad \text{CO}_2 + 2 \text{NH}_4^+$$

$$\text{NADH} + \text{H}^+ \xleftrightarrow{} \quad \text{NAD}^+$$

$\text{NH}_3$ \quad L Glutamate \quad L Glutamate

$\alpha$ Ketoglutarate \quad Dehydrogenase

\begin{itemize}
  \item Figure 5. Urea and ammonium biochemical reactions
\end{itemize}

**Statistics**

Biochemical analyses were presented as means (SD) and range, while results of the quantitative bacteriological cultures were given as box plots displaying the 10th, 25th, 50th (median), 75th and 90th percentiles. In Paper II the ammonium ion production and urea content in the faecal samples of the SIDS infants were compared with those of the control infants using the Mann Whitney U test. Standard Pearson regression analysis was used to determine correlations between the faecal urea content, the faecal ammonium ion production and the faecal NO production. Values $p<0.05$ were considered significant.
**Paper III**

**Geographical and Seasonal Distribution of SIDS**

All of the 636 SIDS cases which occurred in Sweden from 1990 through 1996 was analysed regarding their geographical and seasonal distribution in relation to nitrate concentrations in drinking water and groundwater level changes. Copies of the death certificates were obtained from Statistics Sweden (Statistika Centralbyrå). For the years 1990 - 1996 annual population data from the entire country, for each of the counties and municipalities and also monthly statistics from each municipality regarding births and deaths of infants in the age group 1 - 11 months were provided by Statistics Sweden.

Addresses of the infants recorded on the death certificates were translated to geographical coordinates by the National Land Survey of Sweden; this agency also supplied information regarding source of water supply, whether from a private well or from the municipal water works, to the recorded addresses.

The Geological Survey of Sweden (SGU) matched the geographical coordinates with chemical analyses of groundwater data from its National Groundwater Network records. The SGU record chemical analyses of groundwater twice a year (75 stations) and groundwater levels twice a month (400 stations). These data were used in comparing timing of individual infant deaths with groundwater levels in the county of residence of the infants. The nitrate concentrations of water of private wells (25000 wells) are analysed approximately 1 - 3 times per 10 year period and are used to produce maps of the nitrate concentrations of groundwater; these maps were used to estimate nitrate concentrations of drinking water. The nitrate content of water supplied by the municipal water works are recorded regularly and this information was available to us.

*Statistics*
A spatial scan statistic (SaTScan) developed by Kulldorff (35) was used to detect clusters of rates of SIDS cases in relation to live births in each of Sweden’s municipalities. SIDS cases were assumed to be Poisson distributed and the maximum cluster size assumed to be 50% of the total population. Each geographic centre was considered to be the centre of a potential cluster. Likelihood functions were evaluated using a circle of variable size and location to scan the whole map for areas with high and low rates. Monte Carlo simulation methods were used to approximate the distribution of the likelihood function, and p values for the most likely cluster were determined after compensation for the changing incidence of the disease. Poisson regression analysis was performed to assess if there was a seasonal variation of SIDS cases in different parts of the country and this was analysed both according to the months of the year and according to the quarters of the year.

Chi square tests were used to compare the proportion of days when the groundwater level was high with that when it was low. Groundwater was considered high when it was increasing or at least 75% of the annual maximum level, and low when it was decreasing or below 75% of this level. The expected number of SIDS cases was derived by multiplying the total number of SIDS cases by the proportion of days when the groundwater level was high.

Spearman rank correlation was used to determine if there was a relationship between the local incidence of SIDS and the nitrate concentration in the public water supply.

Significance levels of 0.05 were used throughout the study.

**Paper IV**

**Doubly Labelled Water Technique (DLW)**

This technique is safe and non-invasive with an accuracy of ±5%. It is robust and is especially useful in noncompliant subjects such as infants. This method not only measures carbon dioxide but may be used to derive total energy expenditure, total body water and water
metabolism. However, the cost and the technical expertise required for analysis limit the use of this technique.

**Principle**

An accurately weighed dose of DLW solution containing the stable isotopes deuterium $^2$H and $^{18}$O is given to the subject orally. $^2$H mixes with the water pool while $^{18}$O mixes with both water and bicarbonate pools, which are in equilibrium through the carbonic anhydrase reaction. (36)

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$$

*carbonic anhydrase*

**Figure 6. Carbonic anhydrase reaction**

The rate constant for $^2$H disappearance measures water flux, while the rate constant for $^{18}$O disappearance measures the sum of water and carbon dioxide (CO$_2$) flux; thus the difference between the rate constants gives the CO$_2$ flux. (37)

$$K_D \cong \text{H}_2\text{O flux}$$

$$K_O \cong \text{H}_2\text{O} + \text{CO}_2 \text{ flux}$$

$$\therefore K_O - K_D \cong \text{CO}_2 \text{ flux}$$

*Figure 7. Rate constants for $^2$H ($K_D$) and $^{18}$O ($K_O$)*

$^2$H and $^{18}$O enrichments were determined by mass spectrometric analysis (Micromass, Wythenshawe, UK) of urine samples collected before dosing with DLW (predose) and serially for up to 12 days after dosing. Rate constants were derived from the slopes of lines plotted with isotope enrichment (y-axis) against time (x-axis). (37)

An accurately weighed dose of DLW containing 96 mg $^2$H$_2$O and 240 mg H$_2^{18}$O per kg body weight was given via a nasogastric tube to 8 healthy fullterm infants. Urine samples were
collected prior to DLW dosing (predose) and then following the DLW ingestion, at 4 hours, on day 2, 6, 10 and 12. The samples were collected, using self - adhesive urine bags, and then stored at – 20°C until analysis using the mass spectrometer. (Figure 8)

![Isotope Enrichment Diagram](image)

*Figure 8. Schematic diagram of isotope enrichment plotted against time (days)*

Carbon dioxide production and total energy production were compared between the 2 study periods i.e. with and without ammonium supplementation.

**Ammonium Supplements**

Eight infants were randomized to receive either supplements or no supplements during the first study period (Part A) consisting of 12 days, and vice versa during a second study period (Part B) also of 12 days duration; Part B was performed after a minimum of 2 weeks after completion of Part A.

A sterile water solution containing 500 µmol/mL of ammonium chloride (Addex - Ammoniumklorid ® Fresenius Kabi, Uppsala, Sweden) was prepared. The mothers were instructed on how to measure accurately the assigned doses, to be given to their infants, in 2 mL syringes. An oral dose of 400 µmol/kg/day was given as divided doses of 100 µmol/kg 4 times daily through the 12 days of the study period.
**Biochemical Analyses of Urine**

Urine pH, urea and ammonium concentrations were determined as described previously in Paper I and II. The values obtained following ingestion of ammonium supplements were compared to those when no ammonium supplements were ingested.

**Statistics**

Mean and SE were calculated for age and weights of the infants, carbon dioxide productions, TEE, $^2$H and $^{18}$O dilution spaces and elimination rate constants. Wilcoxon signed rank test and Mann - Whitney U test were used for comparing carbon dioxide productions, TEE, urine urea, ammonium concentrations and pH, with and without ammonium supplementation.
RESULTS

Paper I

Bacteriological Analyses

Anaerobic microflora

_Bifidobacteria_ and _Bacteroides_ were the most prevalent species and were present throughout the first six months of life.

Aerobic microflora

_Streptococci, Enterococci, Escheria coli_ and _Klebsiella_ were the commonest species and were prevalent throughout the first six months.

Faecal pH

The pH of the anaerobic preincubation mixtures was approximately 0.2 pH units lower than that of the aerobic samples, and this remained so throughout the 6 months. The mean (SD) of the pH of the anaerobic and aerobic samples were 5.3 (0.6) and 5.6 (0.8) respectively at 3 days after birth and 5.7 (0.7) and 5.9 (0.6) respectively at six months.

Biochemical Analyses

Faecal urease activity

The faecal urease activity expressed as ammonium ion formation per gram wet weight of faeces after 30 h incubation, decreased steadily from 3 days after birth until six months of age. (Table 1)
<table>
<thead>
<tr>
<th></th>
<th>Aerobic preincubation</th>
<th>Anaerobic preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation urea concentration (mmol/L)</td>
<td>1.95 ± 0.12</td>
<td>1.95 ± 0.12</td>
</tr>
<tr>
<td>3 days</td>
<td>56 ± 68</td>
<td>40 ± 34</td>
</tr>
<tr>
<td>2 months</td>
<td>40 ± 60</td>
<td>36 ± 46</td>
</tr>
<tr>
<td>3 months</td>
<td>36 ± 67</td>
<td>22 ± 26</td>
</tr>
<tr>
<td>6 months</td>
<td>15 ± 14</td>
<td>11 ± 8</td>
</tr>
</tbody>
</table>

Table 1. Faecal urease activity expressed as ammonium ion formed (mmol/g faeces wet weight after 30 h incubation) ± SD in infants from 3 days to 6 months of age, using a low and high urea concentration, under aerobic and anaerobic preincubations.

**Faecal urea content**

The faecal urea content was found to be 5 - 15 μmol/g faeces wet weight from 2 to 6 months of age, and approximately 40 μmol at 3 days after birth. (The water content of the faeces was assumed to be 90%) (Table 2)

<table>
<thead>
<tr>
<th>Age</th>
<th>Urea content μmol/g faeces (wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>39 ± 57</td>
</tr>
<tr>
<td>2 months</td>
<td>4 ± 12</td>
</tr>
<tr>
<td>3 months</td>
<td>5 ± 17</td>
</tr>
<tr>
<td>6 months</td>
<td>6 ± 14</td>
</tr>
</tbody>
</table>

Table 2. Faecal urea content ± SD of infants from 3 days to 6 months of age.

**Nitric oxide concentration (NO)**

The NO concentration in the aerobically preincubated test tubes increased from 52 ± 35 ppb in 3 day old infants to 575 ± 419 ppb (mean ± SD) in 6 month old infants. (Table 3)

NO, sodium nitrate and sodium nitrite, when added to preincubation mixtures were found to have inhibitory effects on faecal urease activity. However this inhibition was not seen when added to sterile urea solutions with jack bean urease. Thus, suggesting that the bacterial production of urease is inhibited and not the urease activity itself.
<table>
<thead>
<tr>
<th>Age</th>
<th>Nitric oxide concentration (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>52 ± 35</td>
</tr>
<tr>
<td>2 months</td>
<td>151 ± 62</td>
</tr>
<tr>
<td>3 months</td>
<td>149 ± 86</td>
</tr>
<tr>
<td>6 months</td>
<td>575 ± 419</td>
</tr>
</tbody>
</table>

*Table 3. Faecal concentrations of nitric oxide ± SD in aerobic preincubated test tubes in infants 3 days to 6 months of age.*

**Paper II**

Out of a total of 30 cases of unexpected infant deaths, 22 were considered to be due to SIDS and 8 due to other causes (pneumonia, shaken baby syndrome, suffocation, septicaemia, subendocarditis, myocarditis). The 8 infants were referred to as controls and were compared with the SIDS infants in the study.

**Bacteriological Analyses**

In both the SIDS cases and in the controls the commonest aerobic microflora were *Enterococci* and *Escheria coli*, while the commonest anaerobic microflora were *Bifidobacteria, Clostridia and Bacteriodes*.

**Biochemical Analyses**

*Faecal urease activity*

The faecal urease activity expressed as ammonium ions formation (mmol/g wet weight of faeces) after 30h at 33°C with urea as substrate at two different concentrations was found to be significantly lower in the SIDS infants compared to the controls. (p<0.05) (Table 4)
Table 4. Mean sigmoid faecal ammonium ion production ± SD after 30h incubation with urea as substrate at two different concentrations.

Faecal urea content and nitric oxide concentrations

The urea content of the faeces of SIDS infants was significantly greater than that of control infants, (p<0.01) while there was no significant differences between the nitric oxide concentrations. (p>0.05) (Table 5)

Table 5. Faecal urea content and nitric oxide concentrations ± SD of SIDS cases compared with control cases.

Paper III

Geographical and Seasonal Distribution of SIDS

In this paper the geographical and seasonal distribution of SIDS in relation to content of nitrate in drinking water and groundwater levels were studied.

Incidence of SIDS and geographical and temporal distribution

The annual incidence of SIDS during 1990 through 1996 decreased from 1.30 to 0.46 deaths per 1000 live births. Maximal incidence of SIDS occurred in the 13th week of life and the median time of death was 11 weeks. The lowest incidence was 0.56 in the Västmanland
region and the highest was 1.15 per 1000 live births in the Västernorrland region. In 79 of the total of 288 municipalities, which accounted for 11% of the total population, there were no SIDS cases during the entire study period.

Seasonal incidence of SIDS and groundwater levels

Majority of SIDS deaths occurred between the months of September and April. Infants born in a certain month were found to have a regional incidence as high as 7 - 8 times the mean incidence for the whole country. The greatest incidence in the north of the country occurred at a time when the south of the country had its lowest incidence. However this was not statistically significant. SIDS cases occurred mostly at a time when groundwater was increasing or high during spring thawing or winter rains. No cases occurred when frost penetration and snow prevented groundwater discharge. The observed timing of deaths was found to be related to high groundwater levels (p<0.001) and more so in small groundwater basins (88% of deaths occurring at high groundwater levels) than in big basins (78% of deaths at high groundwater levels).

Clusters

A statistically significant (p = 0.049) cluster of a high incidence of SIDS comprising 127 municipalities in central and north Sweden occurred during 1990 and 1991. A statistically significant (p = 0.045) negative cluster comprising 33 adjacent municipalities in the counties of Uppsala, Gävleborg, Södermanland, Västmanland and Dalarna occurred during 1995 and 1996. This negative cluster corresponded to a period with a decreasing national incidence as well as low precipitation and low or decreasing groundwater levels in this part of the country.

Incidence of SIDS and the use of private wells

Amongst the SIDS cases, 16.8% had their water supply from private wells; this was similar to that of the live births population, of whom 16.5% had their water supply from private wells. The nitrate concentration (median) in the groundwater of the dug wells (private wells) was
found to be 10 - 20mg/L (range 0.5 - 70) and that of the drilled wells (public water supply) was 1 - 5mg/L (range 0.5 - 70).

**Incidence of SIDS and the nitrate content of drinking water from publicly owned waterworks**

When the incidence of SIDS in a number of municipalities and its correlation to published nitrate concentrations (medians) was studied using a Spearman rank correlation analysis, the correlation coefficient was found to be low (n=53, $r_s=0.07$, $p>0.05$); the correlation was higher when a sample of public waterworks from 1989 which was available at SGU was used (n=30, $r_s=0.74$, $p<0.0001$) or if the chemical content of the drinking water supply of a number of municipalities obtained from records was used (n=18, $r_s=0.48$, $p=0.04$). However, the SIDS incidence was better correlated with published maximal nitrate concentrations (n=53, $r_s=0.34$, $p=0.01$) or if concentrations from a number of public waterworks were used (n=18, $r_s=0.87$, $p=0.0002$). The difference between the published maximal and median nitrate concentrations and the corresponding incidence of SIDS was found to be correlated (n=53, $r_s=0.51$, $p=0.0003$); the correlation was greater using figures from the records of the public waterworks (n=18, $r_s=0.92$, $p<0.0001$). When the maximum concentration and the difference between maximum and median concentrations were plotted against the corresponding incidence of SIDS, the plot of the individual points showed a marked inflection point.

**Paper IV**

**Carbon Dioxide Production**

Carbon dioxide production in infants given 400 $\mu$mol/kg per day of ammonium chloride orally was not significantly different from those of infants who did not receive any supplements. Carbon dioxide production was found to be 0.62 (0.04 SE) mol/kg/d in infants with ammonium supplementation and 0.59 (0.02 SE) mol/kg/d in infants without supplementation. ($p=0.35$)
Total Energy Expenditure

The total energy expenditure values derived from the carbon dioxide production measurements was 0.33 (0.2 SE) MJ/kg/d in infants given ammonium supplements and 0.32 (0.01 SE) MJ/kg/d in infants without supplementation, the difference was not significant.

Urine Urea, Ammonium Ion Concentration and pH

Urea, ammonium ion concentration and pH of the urine of the infants given ammonium supplementation were 50.70 (7.32 SE) mmol/L, 5729 (1334 SE) µmol/L and 6.67 (0.15 SE) respectively. The urea, ammonium ion concentration, and pH of the urine of infants without ammonium supplementation were 49.32 (11.59 SE) mmol/L, 5500 (926 SE) µmol/L and 6.77 (0.17 SE) respectively. The differences between the infants with and without ammonium supplementation were not significant and the p values calculated using the Wilcoxon signed rank test for urea, ammonium ion concentration and pH were equal to 0.46, 0.17 and 0.25 respectively.
DISCUSSION

Urea Metabolism

Urea metabolism is important in the human infant and a derangement of this metabolism could explain some of the factors known to be associated with SIDS. Our results in Paper II show that faecal urease activity is impaired in SIDS resulting in significant amounts of unmetabolized urea in faeces. The low faecal urease activity and the increased faecal urea content differed significantly from that of infants who died of other causes; they are therefore not merely the effects of postmortem decomposition.

Breastmilk is known to contain 3 - 6 mmol/L of urea (increasing in concentration during infancy) and 100 - 500 µmol/L of ammonium ions (decreasing in concentration during infancy). (3, 4) Ingested urea is completely metabolized by enteric microflora (mainly Bifidobacteria, which are urease producing) in the gut of healthy infants. (Paper I) Urea which is thus broken down to ammonium ions is absorbed from the gut and transported via the splanchnic circulation to the liver. Some of the nitrogen (between 17 - 61%) salvaged from the breakdown of urea in the gut is added to the nitrogen pool and may be utilised for protein synthesis. (30, 38, 39) Rate of salvage of urea nitrogen is greatest during the first 6 weeks of life with a gradual slowing down thereafter. (13, 31) In the liver, ammonium is metabolized to urea and carbon dioxide. (33) Urea - synthesizing capacity of the liver matures in term and preterm infants during the first few weeks of life. (40, 41) In Paper IV addition of 400 µmol/kg/day of ammonium ions to the normal diet of breastmilk or formula feeds for 12 days did not augment carbon dioxide production in infants 2 to 6 months old. The dose of ammonium ion given is equivalent to approximately two- and- a- half times the normal intake in a healthy breastfed infant. A much larger oral dose of 2.8 mmol/kg body weight of ammonium chloride is known to cause metabolic acidosis, (42) and this loading dose was used by Adamovich et al to study the effects of metabolic acidosis on renal sodium
reabsorption in neonates. Healthy infants absorb almost 100\% of the oral intake of ammonium present in normal breastfeeding or formula feeds. (Paper I) Infants recovering from infections \((8, 38)\) and low-birth weight infants \((43)\) had greater rates of nitrogen retention than normal infants \((44)\). Compromised infants are thus capable of adapting to their increased nitrogen demands. This could explain the results in our study on healthy infants (Paper IV) Ammonium above the nitrogen needs of the healthy infant may have been lost in faeces or urine. \((45)\) However, urine urea and ammonium in infants given ammonium did not differ significantly from those who were not given ammonium in our study. (Paper IV) Some of the ammonium absorbed may have been incorporated into their body proteins. Heine et al found that between 79 and 94\% of labelled nitrogen instilled in the colon of infants, was retained in their body protein pool. \((11, 12)\)

**Breastfeeding**

Breastfeeding is known to have a protective effect against SIDS. \((46, 47)\) Though modern formula feeds have a low protein content and are enriched with lactose \((8)\) to encourage the growth of *Bifidobacteria* in the intestines, they were unable to suppress the growth of *Clostridia* and *Enterococci*. \((9)\) The urea concentration in formula feeds is equal to that of cow’s milk (i.e. half of that of human breastfeeding). In Paper I the faecal microflora of the infants who were mostly breastfed were found to be predominantly *Bifidobacteria* though aerobic bacteria such as *Enterococci* were present within 3 days of birth. A decrease in *Bifidobacteria* because of an overwhelming increase in pathogenic bacteria or secondary to antibiotic therapy could consequently decrease the breakdown of urea to ammonium ions. In healthy infants the supply of urea and ammonium ions in formula feeds may be adequate. However, in compromised infants, the breastfeeding supply of urea and ammonium ions may be critical.
**Postmortem Vitreous Humor Levels of Urea Nitrogen**

It is interesting to note that postmortem urea nitrogen levels in the vitreous humor of SIDS infants were found to be significantly lower compared to those who had died of other causes. (48) This is consistent with our findings of significant amounts of unmetabolized urea in the faeces of SIDS infants. (Paper II) Postmortem vitreous humor chemistry reflects antemortem blood chemistry and remains stable for up to 30 hours after death. (49)

**Preceding Illnesses**

Gastrointestinal illnesses such as vomiting and diarrhea, are reported by Hoffman (23) and others (24) to occur more frequently in the preceding 2 weeks in SIDS cases than in living control cases; this difference was not found by others. (50, 51) A higher proportion of antibodies to clostridial and enterobacterial toxins and a lower proportion of maternal antibodies were seen in SIDS infants compared to controls suggesting that SIDS infants succumbed to these infections because of an inadequacy in their immune response to these toxins. (52) A change in the normal microflora to a preponderance of pathogenic bacteria could explain the significant amounts of unmetabolized faecal urea seen in SIDS infants. However, we found that faecal microflora in infants who had succumbed to SIDS were similar to those found in normal infants. (Paper I and II)

**Helicobacter pylori Infection**

*Helicobacter pylori* infection has been proposed as a possible cause of SIDS (53) and this has been supported by others. (54) It has been suggested that urease produced by *H. pylori* if aspirated by infants, in whom the gastrooesophageal reflux is commonly seen, could on reaching the alveoli react with plasma urea, to produce ammonia. This ammonia, hypothetically, could be then absorbed systemically producing symptoms of ammonia toxicity resulting in respiratory arrest. (53) On the other hand, absorption of ammonia through the gastric mucosa enables it to be detoxified by the liver. Both *H. pylori* and *Bifidobacteria* are
known to be urease producing and should be able to metabolize completely, the urea contained in breastmilk and formula feeds. If SIDS is caused by a *H. pylori* infection, it does not explain our finding of significant amounts of unmetabolized faecal urea in SIDS infants compared to controls. (Paper II)

**Carbon Dioxide Production and Total Energy Expenditure**

In Paper IV no change in carbon dioxide production was found following ammonium ingestion. The DLW technique was used to determine carbon dioxide production. As this is a non-invasive technique not requiring the cooperation of the subjects, it is well suited for investigations in infants. In a pilot study using the Datex monitor to measure carbon dioxide production following ammonium chloride ingestion difficulties were encountered, as reliable measurements were only possible in the resting or sleeping infant and this was not always the case in the 90 minutes following ammonium ingestion.

Values of total energy expenditure (TEE) can be derived from carbon dioxide production measurements determined by the DLW technique. In 1996 Butte et al (55) published a review of available data of the TEE of infants, assessed by the DLW method, which revealed that estimates of TEE during infancy tended to be lower when obtained by this method than from estimates of dietary energy intake. An extensive data set published in 2000 (56) confirmed the lower values of TEE, determined by DLW method compared to earlier values, which are used in the recommendations for dietary energy by world health authorities such as the FAO. (55, 57) No such data has been published for Swedish infants. In Paper IV we produced these data, which were found to be comparable to that produced by Butte et al, confirming that present recommendations for dietary energy intake are in need of revision.

**Smoking**

Maternal smoking as a potential risk factor for SIDS has been reported as early as 1966 by Steel et al, (58) and this has been confirmed by other researchers. (59, 60, 61) Passive smoke
exposure was also found to increase the incidence of SIDS, (62, 63) and a dose - response relationship between a greater exposure to smoke and SIDS has been reported. (60, 63) Effect of smoke on inflammatory mediators (64) and the presence of nicotine in breastmilk (65) may contribute to the increased risk. Though several reasons have been postulated, no causal mechanism has been elucidated. In Paper I we showed that nitric oxide, nitrate and nitrite have an inhibitory effect on faecal urease, produced by enteric microflora. Nitric oxide present in the ambient air of smokers, when inhaled by infants, could transform to nitrates after dissolving in water, and then impair their enteral urease activity. Inhaled nitric oxide has been demonstrated to be converted to nitrates and subsequently eliminated by the kidneys. (66) Smoking mothers can be presumed to have increased nitrate levels in blood and breastmilk. Inhaled nitric oxide and nitrates in breastmilk may together impair enteral urease activity in the infant resulting in significant amounts of unmetabolized urea and consequently decreasing the amounts of ammonium being transported to the infant liver. The protective effect of breastfeeding was seen in nonsmokers and not in smokers. (63) Endogenous nitric oxide production is known to increase during the neonatal period in normal term infants, and then gradually decrease until the infant is 4 months old, when it reaches a plateau. Preterm infants had greater nitric oxide productions than term infants. (67)

**Prone Position and Body Temperature**

Prone position, hyperthermia and overwrapping have been associated with an increased risk of SIDS in several studies. (68, 69) Decreased ventilation as a result of heat stress has been suggested as the common factor (70) leading to the final apnoea of SIDS. A newborn infant with a low metabolic rate and a large surface area to mass ratio is prone to cold stress, while a 3 month old infant with a higher metabolic rate, lower surface area to mass ratio and better tissue insulation is more prone to heat stress. (71, 72) Thus both cold and heat stress may initiate or enhance hypoventilation especially in an infant with some degree of metabolic alkalosis, which has been suggested to be present in SIDS infants. (34)
Increased Winter Incidence of SIDS

SIDS occurs most frequently in the winter months in both the northern and the southern hemispheres. Infection, (73) overwrapping, and smoking in confined spaces have all been previously suggested to contribute to the increased winter incidence. Peak levels of environmental pollutants such as carbon monoxide, sulphur dioxide, nitrogen dioxide and hydrocarbons have been shown to precede the increase of SIDS. (74) Animal studies have demonstrated that nitrogen dioxide resulted in changes in the bronchial epithelium which contributed to a susceptibility to infection. (75, 76) Prone sleeping partially accounted for the increase in SIDS rates in winter but some unidentified seasonal factor was found to modify its effect. (77)

In Paper III the seasonality of SIDS during 1990 through 1996 in Sweden was found to vary according to the location of the infants’ residences, with reference to the geographical latitude. The incidence of SIDS was highest in the northernmost part of the country when it was at its lowest in the rest of the country. The occurrence of individual deaths was associated with the recharge of groundwater, which is known to increase its nitrate content. (78, 79, 80) SIDS cases occurred when the groundwater level rose, mostly as a result of spring thawing or autumn rains. No SIDS cases occurred in the northernmost parts of the country, when frost penetration and snow prevented changes in the groundwater level. This is in agreement with a study by Munro et al (81) who showed that infant mortality was more than 31 percent greater in infants resident in waterlogged soils than on well drained soils. Change in the environmental temperature rather than the exact temperature was found to be related to the incidence of SIDS. (82) Greatest risk of SIDS was on the warmer days of winter when the daytime average was 3°C greater than the preceding 30 days. (83) Thawing on the warmer days following a colder spell with a resultant increase in water nitrate levels could be a possible explanation.
CONCLUSION

1. Aerobic and anaerobic microflora appear in the faeces of healthy infants within 3 days of birth and are present throughout the first 6 months of life. Faecal urease activity decreases to a third while bacterial production of nitric oxide increases ten-fold during the same period. Nitrate, nitrite and nitric oxide have an inhibitory effect on faecal urease activity.

2. An insufficient metabolism of enteric urea is associated with SIDS.

3. The seasonal distribution of SIDS varies widely in different parts of the country and is associated with groundwater level changes. Peak or widely varying drinking water nitrate concentrations is associated with the incidence of SIDS.

4. Ingestion of additional ammonium chloride, equivalent to at least two-and-a-half times the normal amount found in breastmilk feeds, does not increase carbon dioxide production in healthy infants.

Inadequate enteric urea and nitrogen metabolism in infants and peak nitrate levels in drinking water are associated with the incidence of SIDS. Further evidence is required to demonstrate if they play a causal role in SIDS.

Future Research

Several questions remain to be answered.

1. Do infants of smoking mothers have an impaired faecal urease activity and a high faecal urea content compared to controls?

2. Are nitrate levels in drinking water in countries known to have a high incidence, greater and more varying than those of countries with a low incidence?

3. Do infants at a high risk of SIDS have a metabolic alkalosis?
4. Does the addition of ammonium supplements to the feeds of low-weight infants and infants with infections, increase their carbon dioxide productions?
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