**Relevant Excerpts from the Grant Application**
**(face page, pages 2-3, and pages 8-18)**

**(02-0898)**

**Dr. Kimberly Spence**

**Title: Precursor Preference in Surfactant Synthesis of Newborns**

AA PART I *(Form Pages 1 to 6, 9)*

Form Approved through 8/31/01
OMB No. 0925-0002

Department of Health and Human Services
Public Health Service

# Individual National Research Service Award Application

*Follow instructions carefully.*
*Do not exceed character length restrictions indicated on sample.*

| LEAVE BLANK—For PHS use only | | |
|---|---|---|
| Type | Activity | Number |
| Review Group | | Formerly |
| Meeting Dates | | Date Received |

1. TITLE OF RESEARCH TRAINING PROPOSAL *(Do not exceed 56 characters, including spaces and punctuation.)*
Precursor Preference in Surfactant Synthesis of Newborns

2. LEVEL OF FELLOWSHIP
Postdoctoral

3. PROGRAM ANNOUNCEMENT/REQUEST FOR APPLICATIONS

4a. NAME OF APPLICANT *(Last, first, middle initial)*
Spence, Kimberly L.

4b. E-MAIL
Spence_K@kids.wustl.edu

4c. HIGHEST DEGREE(S)
M.D.

4d. PRESENT MAILING ADDRESS *(Street, city, state, zip code)*
Washington University
Pediatrics-Newborn Medicine
660 South Euclid Avenue
Campus Box 8116
Saint Louis, MO 63110-1093

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Saint Louis, MO 63110-1093

4f. OFFICE TELEPHONE NO. *(Area code, no., and ext.)*
(314) 454-2683

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4h. PERMANENT PHONE NO. *(Area code and no.)*
(314) 454-2683

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(314) 454-4633

4j.
[X] U.S. CITIZEN OR U.S. NONCITIZEN NATIONAL *or* [ ] PERMANENT RESIDENT OF U.S.

5. TRAINING UNDER PROPOSED AWARD *(See Lexicon)*
Discipline No. 360 Category Name Other Clinical Medicine

6. PRIOR AND/OR CURRENT NRSA SUPPORT *(Individual or Institutional)*
[X] NO [ ] YES *(If "Yes," refer to Item 24, Form Page 5)*

7a. DATES OF PROPOSED AWARD
From (MM/DD/YY) 5/1/03 Through (MM/DD/YY) 4/30/05

7b. PROPOSED AWARD DURATION *(in months)*
24 months

8. DEGREE SOUGHT DURING PROPOSED AWARD
Degree Expected Completion Date

**SPONSOR COMPLETES ITEMS 9 through 14**

9. HUMAN SUBJECTS
[ ] NO [X] YES
9a. If "Yes," Exemption No. *or* IRB Approval Date 10/9/02 [X] Full IRB *or* [ ] Expedited Review

9b. Assurance of Compliance No.
FWA00002284

10. VERTEBRATE ANIMALS
[X] NO [ ] YES
10a. If "Yes," IACUC approval date
10b. Animal Welfare Assurance No.

11a. NAME OF SPONSOR *(Last, first, middle initial)*
Hamvas, Aaron
Telephone (314) 454-6148
Fax (314) 454-4633
E-mail Address hamvas@kids.wustl.edu

11b. NAME OF PROPOSED SPONSORING INSTITUTION
Address Washington University
660 South Euclid Avenue
Saint Louis, MO 63110-1093

11c. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Pediatrics

11d. MAJOR SUBDIVISION
School of Medicine

12. ENTITY IDENTIFICATION NO. 1430653611A1
DUNS NO. *(if available)* 06-855-2207

13. NAME AND TELEPHONE NO. OF ADVISOR IF DIFFERENT FROM 11a.
Telephone

Name and address of institution where research training will take place if different from Item 11b.

Address

14. NAME OF OFFICIAL IN BUSINESS OFFICE
Teri Love
Telephone (314) 362-6150
Fax (314) 362-0315
Title Manager, Grants & Contracts
Address Washington University
660 South Euclid Avenue
Saint Louis, MO 63110-1093

E-mail Address ggcinfo@msnotes.wustl.edu

15. APPLICANT CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and I agree to comply with the Public Health Service terms and conditions if an award is issued as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I certify that I have read the National Research Service Award Service Assurance, that I will abide by the Assurance if an award is made, and that the award will not support residency training.

SIGNATURE *(Required of each applicant)* Kimberly L. Spence, M.D. DATE 11/26/02

## Individual NRSA Application

*(To be completed by applicant—follow PHS 416-1 instructions)*

NAME *(Last, first, middle initial)*: Spence, Kimberly L.

16. APPLICANT'S EDUCATION

| DEGREE | MONTH | YEAR | FIELD | INSTITUTION | MENTOR |
|---|---|---|---|---|---|
| BA | 05 | 1994 | Biochemistry | Connecticut College | |
| MD | 05 | 1998 | Medicine | University of Missouri | |

17. APPLICANT'S TRAINING/EMPLOYMENT *(After college)*

| ACTIVITY/ OCCUPATION | BEGINNING DATE (mm/yy) | ENDING DATE (mm/yy) | FIELD | INSTITUTION/COMPANY | SUPERVISOR/EMPLOYER |
|---|---|---|---|---|---|
| Pediatric Resident | 7/98 | 6/01 | Pediatrics | St. Louis, Children's Hospital | Aaron Hamvas, M.D. |
| Clinical Fellow | 7/01 | Present | Pediatrics<br>Newborn Medicine | Washington University | Aaron Hamvas, M.D. |

Board Certification - 1/02 - Pediatrics

18. GOALS FOR FELLOWSHIP TRAINING AND CAREER

There are two goals of my fellowship training. The first goal is to learn to care for patients. The second goal is to learn to conduct scientific and clinical research at an academic institution. This project will serve as an introduction to translational medicine and thus help establish my career path as a physician scientist. While learning to conduct research I hope to improve my skills in a variety of areas including: 1) project planning and execution, 2) scientific methods such as chromatography and mass-spectrometry, 3) data collection and statistical analysis, and 4) scientific paper writing and reviewing. I hope to continue my role as a physician scientist in my future career as a neonatologist.

### SPONSOR

19. NAME AND DEGREE(S): Aaron Hamvas, M.D.

20. POSITION/RANK: Professor of Pediatrics (Newborn Medicine Division)

21. RESEARCH INTERESTS/AREAS: Utilization of stable isotopes and molecular genetic analysis to identify and understand inherited disorders of pulmonary surfactant metabolism.

### RESEARCH PROPOSAL

22. DESCRIPTION (Do not exceed space provided)

Pulmonary surfactant, a mixture of phospholipid and protein, stabilizes lungs by lowering surface tension and preventing alveolar collapse at end expiration. Respiratory distress syndrome (RDS) in infants most commonly results from a quantitative deficiency of pulmonary surfactant after premature birth (1,2). Nutrition may affect the composition and function of pulmonary surfactant (3). It is unclear if preformed fatty acids or de novo synthesized fatty acids are utilized preferentially in preterm infants for surfactant phospholipid synthesis. Recently developed methods utilizing naturally occurring, stable, non-radioactive isotope labeled metabolic precursors of phospholipid synthesis provide the opportunity to understand possible influences on surfactant metabolism in infants (4-7). We will use intravenous infusions of surfactant phospholipid precursors ( [1,2,3,4-13C4] palmitate and [1-13C1] acetate) and gas chromatography/mass spectrometry (GC/MS) to test the hypothesis that: plasma palmitate is utilized preferentially for surfactant synthesis in preterm infants less than 28 weeks gestational age. The respective rates of acetate and palmitate incorporation into the surfactant of preterm infants will be compared. The use of labeled metabolic precursors of surfactant phospholipid provides a unique and powerful approach in the evaluation of surfactant metabolism.

| Individual NRSA Application<br>**Table of Contents** | NAME *(Last, first, middle initial)*<br>Spence, Kimberly L. |
|---|---|

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**Section 3—References** *(Minimum of 3)*
*(See instructions for submission of references)*
List full name, institution, and department of individuals submitting reference letters.

| | | |
|---|---|---|
| F. Sessions Cole, M.D. | Washington University | Department of Pediatrics |
| Alan L. Schwartz, Ph.D., M.D. | Washington University | Department of Pediatrics |
| Bruce W. Patterson, Ph.D. | Washington University | Department of Internal Medicine |

Other Items *(list):*
Personal Data Page for Fellows

**Section 4—Appendix**
*(3 collated sets. No page numbering necessary. Not to exceed 3 publications; 2 for predoctoral candidates.)*
[X] Check if Appendix is included

## 30. RESEARCH TRAINING PLAN

### 30a. Approximate Percentage of Proposed Award Time

First year:
Didactics including coursework 10%
Research 90%

Second Year
Clinical time 10%
Research 90%

| Sponsor/ course | Subject material | Schedule |
|---|---|---|
| Program in Translational and Experimental Medicine (ProTEM) at Washington University School of Medicine. | Provides a framework for research training for fellows from all disciplines. Formal courses through this program include biostatistics, epidemiology, and experimental design as well as training in grant and manuscript writing and research presentations. | Classroom 90 minutes a week plus independent study |
| Introduction to Statistics for the Health Sciences | statistics | |
| Division Newborn Medicine | ethics, statistics, methods in critical appraisal of the literature, and the practice of evidence-based medicine | 120 minutes per week |
| Department of Pediatrics | research forum and didactic series for the fellows in ethics, statistics, methods in critical appraisal of the literature, and the practice of evidence-based medicine | 60 minutes per week |

### 30b. Research Training Proposal

#### 1. Specific Aims

**Specific Aim: 1) To determine the rate of surfactant phospholipid production from preformed fatty acids (palmitate) in preterm infants less than 28 weeks gestational age at:**

a. birth
b. 2 weeks of life
c. 4 weeks of life.

**Specific Aim: 2) To determine the rate of surfactant phospholipid production from de novo synthesis of fatty acids (acetate) in preterm infants less than 28 weeks gestational age at:**

a. birth
b. 2 weeks of life
c. 4 weeks of life.

**We will measure the rate of incorporation of $^{13}C$ into surfactant obtained from tracheal aspirate samples after simultaneous 24 hour infusions of [1-$^{13}C_1$] acetate and [1,2,3,4-$^{13}C_4$] palmitate. The**

understanding of the unique substrate needs for critically ill newborns will permit more specific interventions, including possibly nutritional supplementation, to be developed.

## 2) Background and Significance

*a) Surfactant and its production*

The phospholipid components of pulmonary surfactant are synthesized from plasma fatty acids and de novo synthesized fatty acids in liver, adipose tissue, and Type II cells. The surfactant phospholipids are composed of a glycerol backbone, two fatty acids and a polar phosphorylated moiety (8). Phosphatidylcholine (PC) comprises approximately 80% of the phospholipid component of pulmonary surfactant and is the primary contributor to the surface tension lowering properties of surfactant. Approximately 60% of the surfactant PC molecules contain two saturated fatty acid residues (disaturated PC), most of which are palmitic acid, which is thus known as dipalmitoyl phosphatidylcholine (DPPC). Whether the palmitate in DPPC is synthesized de novo from acetate or other precursors, or is incorporated directly from plasma palmitate is unknown in humans. De novo synthesis refers to the synthesis of DPPC from acetate as the precursor. Another possible synthetic pathway is the use of a preformed fatty acid such as plasma palmitate as a precursor. Animal studies have yielded conflicting results. Jobe et al used radiolabeled acetate and palmitate in adult, 3 day old and newborn rabbits to compare incorporation of acetate versus palmitate into surfactant phospholipids (9). Jobe interpreted the results to indicate "the type II pneumocyte may use acetate preferentially for the synthesis of" palmitate (9). Sato and Akino also found that acetate was the preferred substrate for surfactant synthesis in rats (10). In contrast Martini found that palmitate or preformed free fatty acids are the primary precursors for surfactant PC synthesis in adult pigs (6). Our lab has used stable isotope labeled glucose and acetate to investigate surfactant production from de novo synthesized fatty acids in newborns to be described in Section 2c (11,12). The opportunity to simultaneously infuse [1,2,3,4-$^{13}C_4$] palmitate and [1-$^{13}C_1$] acetate will permit more specific understanding of surfactant metabolism which will then lead to more specific therapies for preterm infants with RDS.

*b) Potential impact of nutrition and injury on surfactant composition and function*

Wolfe et al. studying adult pigs found that diets low in palmitate resulted in a reduced amount of DPPC in lung surfactant and decreased pulmonary function (3). Compliance was reduced in the pigs fed predominantly fats containing linoleate and fish-oil compared with the palmitate supplemented group. Lung compliance deteriorated in all three groups when the pigs were stressed with endotoxin. However, the lung compliance of the palmitate group was not as compromised suggesting that lung injury could influence surfactant composition and function. Thus nutritional intervention might be a possible venue to explore in the management of lung disease in the premature infant. In addition Martini et al found that thermal injury in pigs decreased the PC pool and the relative proportions of palmitate in the PC (13).

Nothing is known about substrate utilization in preterm humans. This proposal utilizing stable isotope labeled precursors of surfactant offers for the first time the ability to evaluate this question.

c) *Stable isotope methods*

Stable isotopes have been utilized for decades to study in vivo protein, amino acid, carbohydrate and lipid metabolism, and can now provide the opportunity to study surfactant metabolism in vivo in human neonates (4,5,7,14,15). Naturally occurring, non-radioactive, stable isotopes of carbon ($^{13}C$) are administered as metabolic precursors of surfactant synthesis and measured in surfactant extracted from tracheal effluent with gas chromatography-mass spectrometry (GC/MS) (4,5). Figure 1 is a representative enrichment curve for $^{13}C$ in surfactant PC obtained from one infant after [U-$^{13}C_6$]glucose administration. From this curve, the time to appearance of label in PC (Tapp measured in hours), the time to maximum enrichment (Tmax measured in hours), the maximum enrichment (Emax represented by the tracer:tracee ratio of tracer labeled palmitate versus unlabeled palmitate in surfactant), the half life of clearance ($T_{1/2}$ measured in hours), and the fractional synthetic rate (FSR measured as %) of PC from glucose are calculated, as described in the Figure 1 legend.

The fractional synthetic rate (FSR) is the relative contribution of a precursor to the newly synthesized surfactant pool.

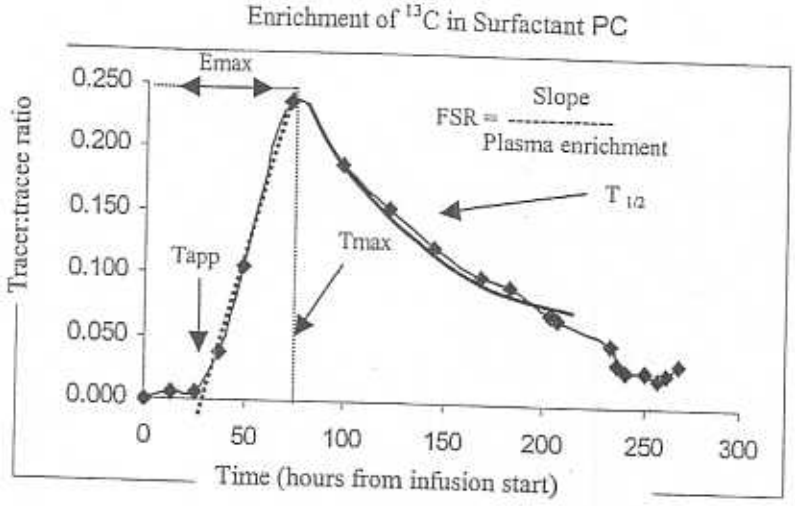



Fig. 1. From the ascending limb of the curve, linear regression was used to calculate the time of appearance for $^{13}C$ in surfactant PC ($y=0.005x-0.132$, $r^2=0.99$), 26.4 hours in this example. By dividing the slope of the curve by the steady-state enrichment of $^{13}C$ in the plasma (3.0%), the fractional synthetic rate was calculated: (0.005/3.05) x 24 = 3.9% in 24 hours. From the descending limb of the curve, monoexponential curve fitting was used to calculate the half-life of clearance of labeled PC ($y=0.678e^{-0.012x}$, $r^2=0.94$), 57.8 hours in this example.

Some of the currently available studies of surfactant metabolism in humans utilizing stable isotopes are summarized in Table 1. Three studies in animals have provided information about the validity of these methods in humans. As discussed above Martini et al. in young normal pigs found that the FSR was 36% per day for plasma palmitate, but only 3% per day for PC synthesized *de novo* from acetate (6). This study required removal of the lungs to determine tracer enrichment in the intracellular and extracellular compartments. In the Bunt and Janssen studies in premature baboons, the $^{13}C$ enrichment in PC obtained simultaneously from tracheal aspirate, alveolar wash, and lung homogenate was similar, suggesting that PC palmitate isolated from tracheal aspirate was an appropriate reflection of alveolar or tissue surfactant (16,17).

Table 1. Summary of stable isotope studies in surfactant metabolism

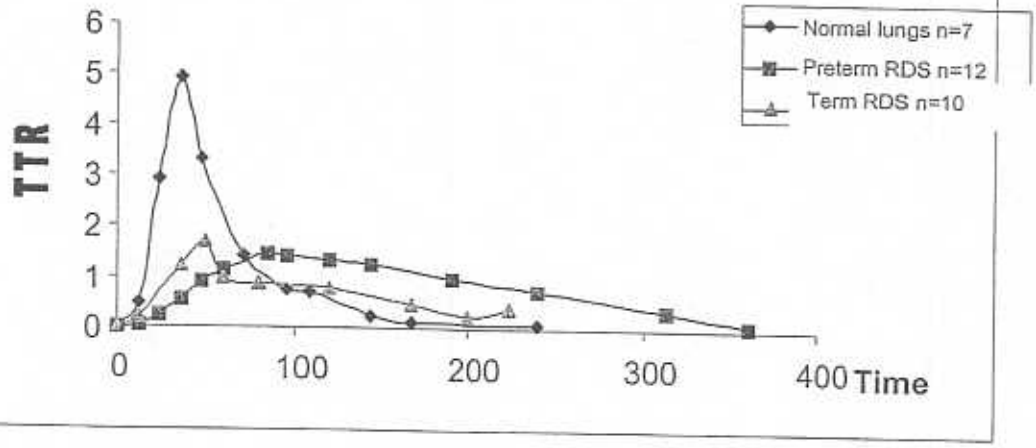
| Study | Population | tracer | Tapp (h) | Tmax (h) | $T_{1/2}$ (h) | FSR |
|---|---|---|---|---|---|---|
| Bunt (4) | Preterm humans | [U-$^{13}C_6$] glucose | 19 (2) | 70 (18) | 113 (24) | 3% |
| Cogo (5) | Human infants <6mo | [U-$^{13}C_{16}$] palmitate | 9 (5) | 49 (9) | 17-178 | 34% |
| | | [U-$^{13}C_{18}$] linoleate | 10 (7) | 46 (19) | 37-144 | 50% |
| Merchak (18) | Preterm human | [U-$^{13}C_6$] glucose | 13-15 | 70-87 | 76-79 | 4.2-4.7 |
| Merchak (12) | Preterm human | [U-$^{13}C_6$] glucose | 12 | 47 | | 3% |
| Bohlin (19) | Term human<br>1)Normal lungs<br>2) Lung disease<br><br>Preterm | [1-$^{13}C_1$]acetate | 7<br>9-11<br>13 | 35<br>46-53<br>87 | 28<br>38-63<br>106 | 15%<br>3-13.8%<br>2.2% |

In these human studies, [U-$^{13}C_6$] glucose, [U-$^{13}C_{16}$] palmitate or [U-$^{13}C_{18}$] linoleate were administered intravenously to evaluate three measures of surfactant production ($T_{app}$, $T_{max}$, and the FSR) and one measure of surfactant clearance ($T_{1/2}$). No previous study has investigated the relative contribution of de novo synthesized fatty acids versus preformed plasma fatty acid to surfactant production in newborns.

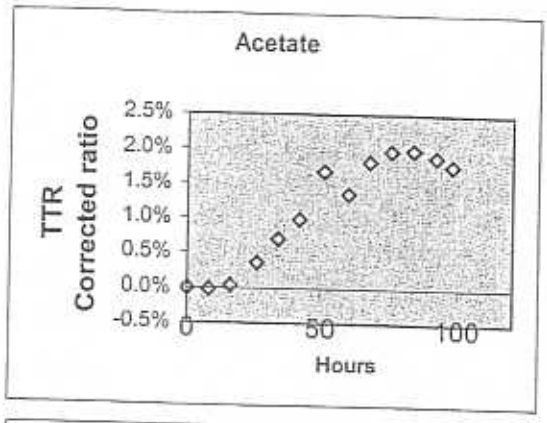
These small human studies have yielded results comparable to those of the animal studies and thus demonstrate the feasibility of using stable isotopes to evaluate surfactant production and catabolism. In the proposed studies we will use simultaneous infusions of [1-$^{13}C_1$] acetate and [1,2,3,4-$^{13}C_4$] palmitate, specifically chosen to be able to differentiate surfactant produced from de novo synthesis of fatty acid (acetate) versus that produced from preformed plasma fatty acids (palmitate). GC/MS distinguishes the surfactant phospholipid derived from acetate and palmitate on the basis of molecular mass differences - one additional mass unit (m+1) for that derived from [1-$^{13}C_1$] acetate (with a single $^{13}$C-atom) and 4 additional mass units (m+4) for that derived from [1,2,3,4-$^{13}C_4$] palmitate (with four $^{13}$C-atoms).

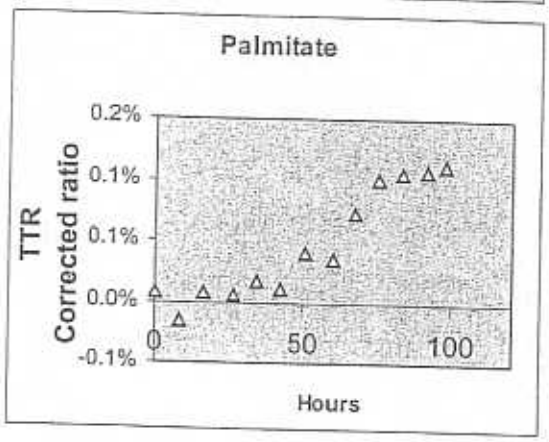
The Division of Newborn Medicine at Washington University has extensive experience with these stable isotope methods to evaluate surfactant metabolism. Over 60 infants have been studied to date at St. Louis Children's Hospital/ Washington University. The current methods as outlined have been able to demonstrate differences in surfactant metabolism among different subsets of infants including premature infants less than 28 weeks gestation, term infants with lung disease and term infants without lung disease (Figure 2). These studies were performed with [1-$^{13}C_1$]acetate. The calculated indices from these curves are displayed in Table 1 (Bohlin) and described in a manuscript that has been submitted for publication (Appendix).

**Fig 2. Enrichment curves based on group**




In my laboratory time to date during my fellowship, I have begun to use the methods described above to address the hypothesis of this proposal. Figures 3 and 4 are representative time-enrichment curves from a 2 day old infant of 25 weeks gestation who received simultaneous infusions of [1-$^{13}C_1$] acetate (Figure 3) and [1,2,3,4-$^{13}C_4$] palmitate (Figure 4). The accompanying table displays the calculated metabolic indices from each precursor.




| Table 2 | Acetate | Palmitate |
|---|---|---|
| Tapp | 14.4 hrs | 25 hrs |
| FSR | 0.03 pools/day | 0.11 pools/day |
| Tmax | 84 hrs | 98 hrs |

The remarkable feature of this preliminary study is the difference in FSR's for acetate and palmitate. This preliminary study demonstrates the feasibility of conducting this study and as well as the interpretability of the results. $FSR_{acetate}$ represents the fractional rate of synthesis from de novo formation of surfactant palmitate from acetate, and $FSR_{plasma\ palmitate}$ represents the fractional rate of synthesis from plasma palmitate.

While this is only one study, the results are consistent with our hypothesis. As expected the $FSR_{palmitate}$ is higher than $FSR_{acetate}$, reflecting preferential incorporation of palmitate versus acetate.

## 3) Research Design and Methods

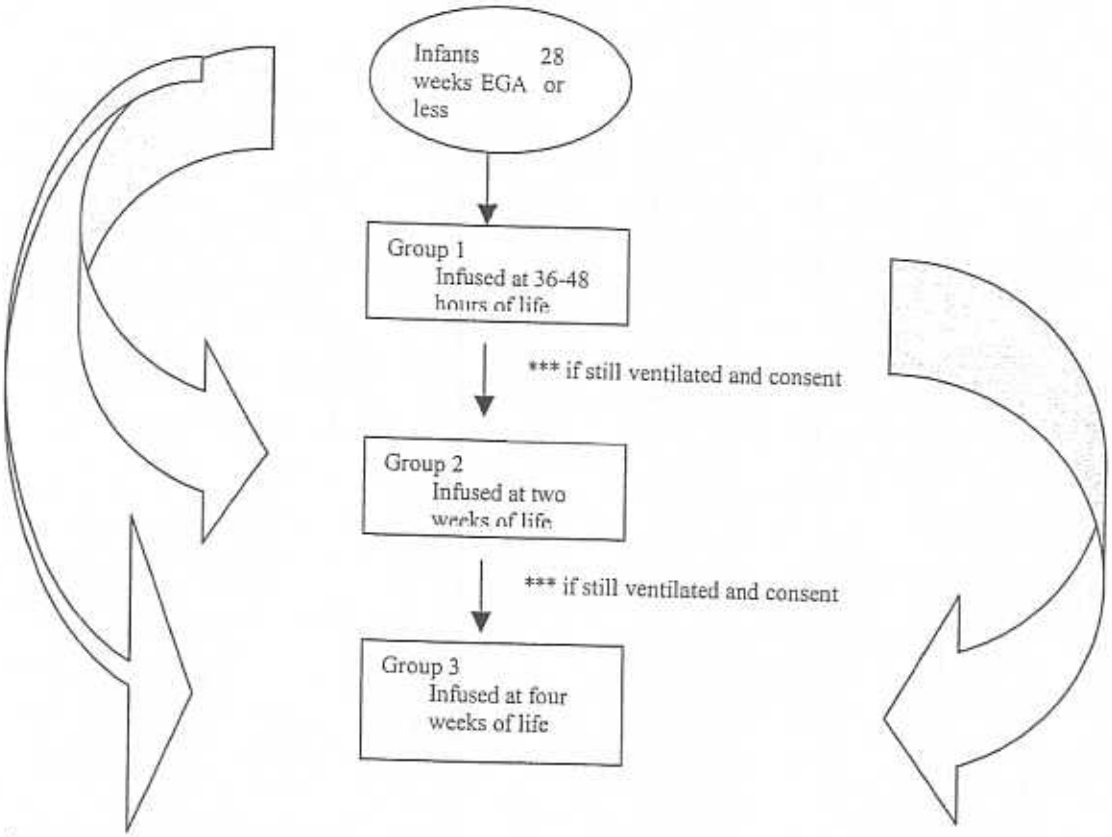
1. *Participation of Children*

These studies will enroll children only, specifically infants less than twenty-eight weeks post-conceptual age at: 1) at birth, 2) two weeks of life and, 3) four weeks of life. A fourth group of infants without lung disease will also be studied and serve as controls. The eligibility criteria for group 4 include: 1) less than 6 months of age, 2) any gestational age, 3)airway access for at least 5 days, 4) no lung disease ie. on room air with a normal CXR.

After consent is obtained from the patent's medical care team and parents, infants will be enrolled in the study. For Group 1, the study will start twenty-four hours after the last dose of surfactant. At the start of the study baseline blood and tracheal effluent samples will be obtained. Infants will then receive simultaneous 24 hour intravenous infusions of [1,2,3,4-$^{13}C_4$] palmitate and [1-$^{13}C_1$] acetate. Should indwelling vascular access be available for blood drawing a total of five blood samples will be obtained at time 0, 8, 16, 24 and 27 hours post-initiation of infusion. Otherwise blood will be drawn in conjunction with clinically indicated specimens. Tracheal effluent samples will be collected at the same times and then every 8 hours thereafter for a week followed by collection every 12 hours for the last week. The surfactant will be isolated from the samples and analyzed with the GC-MS. Indices will be calculated as in the methods section.

2. *General overview of research plan*
The following diagram demonstrates the recruiting techniques and study design.

**Figure 5**




As the block arrows indicate in the flow diagram, patients who do not undergo a study infusion in Group 1 may participate in Group 2 and/or 3 and patients not eligible for Group 1 or 2 may participate in Group 3.

Demographic data, including maternal corticosteroid administration, birthweight, gestational age, race and sex will be obtained from each medical record. Data about clinical characteristics that may influence surfactant metabolism will be linked with the surfactant metabolic data to explore relationships between the kinetics of surfactant metabolism and the clinical expression of pulmonary dysfunction. These data include the type, dégree and duration of mechanical ventilation, blood gas analyses, fluid, glucose, protein and lipid infusion rates, and corticosteroid administration. If an infant dies within 2 weeks of undergoing a stable isotope infusion, autopsy consent will be sought in order to obtain bronchoalveolar lavage fluid and lung tissue.

3. *Patient population*
Premature infants: Infants who are admitted to the Saint Louis Children's Hospital Neonatal and Pediatric Intensive Care Units with the following inclusion criteria will be eligible for participation: 28 weeks gestational age or less (by best obstetrical dating), clinical and radiographic signs of respiratory distress syndrome (RDS) and need for mechanical ventilation. Infants of both sexes and all ethnic groups will be eligible to participate. Exclusion criteria include imminent death, known infection, congenital anomalies, fetal hydrops and pulmonary hemorrhage. We chose premature infants of 28 weeks or less gestation for several reasons based on the St. Louis Children's Hospital NICU experience. First to obtain sufficient kinetic data, it is necessary that the infant remain intubated for at least 5-7 days in order to obtain tracheal effluent samples. Infants >28 weeks gestation may not require mechanical ventilation for that duration. Also the number of eligible infants admitted to the NCIU is about 125/year.

The control infants as outlined in the inclusion criteria above will be 6 months chronological age or less. Potential candidates include infants with heart disease ventilated for surgery or infants with congenital anomalies undergoing surgery. Because of the limited population of infants with normal lungs who have airway access for a sufficient period of time, the control infants will not be matched for gestational or chronological age. The likely differences in gestational age may complicate the interpretation of "normal" substrate utilization but will not detract from understanding substrate utilization in critically ill preterm infants.

4. *Specific methods:*

*Isotope infusions*

The [1,2,3,4-$^{13}C_4$] palmitate and [1-$^{13}C$] acetate are prepared in a solution together to provide 58 umol/kg palmitate and 3 mmol/kg acetate in 5% albumin and 4% dextrose over 24 hours. This infusion will provide 24 ml/kg volume and approximately 3.7 mmol/kg sodium per day (maintenance needs are 3-5 mmol/kg per day). This extra fluid, sodium and acetate will be factored into the electrolyte, glucose and fluid requirements for the infant during the day of the infusion. The fat infusion will stay constant during the infusion.

*Tracheal aspirate analysis*

Tracheal aspirate surfactant extraction: Tracheal aspirate samples are processed to yield disaturated phospholipids (which are specific for pulmonary surfactant) by a modification of the method of Mason et al. (20). After thawing, the tracheal aspirates are centrifuged to remove cell debris, the supernatant is removed. To determine the absolute amount of disaturated phospholipids in the sample and also to determine the recovery rate of phospholipid through the processing steps, 0.101 μmol of C17:0 phosphatidylcholine is added. After a chloroform:methanol extraction, 150 μl of an osmium tetroxide solution (20mg osmium tetroxide in 1ml chloroform) is added to the supernatant and passed through a neutral alumina column. To determine the absolute amounts and the composition fatty acids in the surfactant, the eluate is derivatized with 10% acetyl chloride in methanol. This mixture is incubated at 70°C for 30 minutes, dried, and 100 μl heptane is added to dissolve fatty acid methyl esters. Quantitative GC is performed on a Hewlett Packard 5890 instrument using a 30 x 0.32 mm omegawax 320 column (Supelco) with a flame ionization detector. The isotopic enrichment of $^{13}C$ in palmitate is measured in the saturated phospholipids by gas chromatography/mass spectrometry (GC/MS, Hewlett-Packard Model 5973, Palo Alto, CA, USA).

*Plasma palmitate analysis*

To measure palmitate precursor enrichment, plasma lipids are extracted with hexane, methyl esters of free fatty acids prepared by reaction with iodomethane, and fatty acid methyl esters separated from other lipids by solid phase extraction over silica cartridges (21). A known quantity of C17:0 fatty acid is added to plasma prior to sample preparation to quantify plasma fatty acid concentrations by quantitative GC analysis, using a 30 x 0.32 mm Omegawax 250 column with a flame ionization detector. Quantitative GC analyses are standardized using a fatty acid methyl ester standard of known composition.

*MIDA*

To measure acetate precursor enrichment, mass isotopomer distribution analysis (MIDA) will be used. With MIDA, the relative proportions of newly synthesized palmitate containing one and two labeled acetate precursors (singly and doubly labeled palmitate, respectively) is evaluated, from which the isotopic enrichment of the direct biosynthetic precursor pool (acetate), and the fractional rate of synthesis from acetate (i.e. de novo synthesis) can be determined.

*Hazardous Materials*

Materials to be used that are hazardous include methanol, chloroform, ammonium hydroxide, acetyl chloride and osmium tetroxide. These are all handled in a certified fume hood wearing gloves and safety glasses.

*Data analysis*

Paired comparisons (paired t-tests or Wilcoxon signed rank tests) will assess differences in FSR between acetate and palmitate. Unpaired analyses (t-tests for normally distributed data or Wilcoxon rank sum analyses for non-normally distributed data) will be used to assess differences between groups. For infants with sequential infusions, paired comparisons will again be used to assess changes in precursor utilization over time. These data will help answer the questions of whether the indices change and/or precursor preference changes with age or disease progression. Further sub-group analyses will include stratification by race and gender. The power to detect group differences in FSR from previous studies has suggested that a sample size of 10 infants at each time point will yield at least 80% power (at alpha = 0.05) for a 2-tailed test.

Comparisons utilizing clinical data, including pre- and postnatal corticosteroid exposure, degree and duration of ventilatory support, survival to 1 month of age and beyond, etc. will be performed to identify possible influences of disease severity or interventions on precursor incorporation. All data will be entered into a database and analyzed with the SAS system for personal computers (SAS Systems, Cary, NC).

*Formal Educational Plan*

Please see Item 30a. In brief summary I will be enrolling in a course entitled "Introduction to Statistics for the Health Sciences." It is a basic course in statistics to be started in the spring of 2004. There is also a course in translational medicine, which I plan to start during the fall of 2003.

**Participation of Children**

*(1) Involvement*

Neonatal respiratory distress affects infants of both genders and those of all ethnic groups. Therefore, all infants, regardless of gender or race, will be eligible to participate in these studies.

Premature infants will be eligible to participate in this research. We will enroll 30-40 infants over the 18 month study period. They will all be critically ill and hospitalized in the neonatal intensive care unit at St. Louis Children's Hospital/Washington University Medical Center. The major aspect of this study is performed for research purposes only and includes the intravascular infusion of the stable isotopes.

The blood draws will include an extra 2.5 ml of blood. The blood withdrawn for the study is minimal in comparison to the amount drawn for routine clinical purposes is not likely to contribute to the need for additional transfusions. The tracheal aspirate samples will be obtained at the time of suctioning of the infant's endotracheal tube, which is part of standard pulmonary toilet.

Inclusion criteria: Only infants who require mechanical ventilation as part of the routine management of their illness will be eligible. One group of infants will be studied: premature infants 28 weeks' gestational age and less.

Exclusion criteria: Infants for whom death appears imminent, those with known infection, congenital anomalies, fetal hydrops, and pulmonary hemorrhage.

*(2) Sources of research material*

The specimens to be obtained from identifiable subjects include blood and tracheal aspirate, along with clinical data from the medical record. The blood samples will be obtained specifically for research purposes. The tracheal aspirate samples would be obtained as part of routine clinical care. However, instead of being discarded the aspirates will be saved for analysis.

*(3) Recruitment procedures*

Admissions to the neonatal intensive care unit and pediatric intensive care unit at St. Louis Children's Hospital will be monitored daily to identify candidates for any of the studies. The principal investigator or designee will discuss an infant's eligibility with the attending physician. Following assent from the attending, the parents will be approached in person for consent. The parents will be told that this study entails administration

of non-radioactive isotopes that are normal constituents of the intravenous fluids that their child will receive routinely as part of his/her care. In addition they will be told that about 1/2 of a teaspoon of extra blood will need to be drawn. It will be drawn from a vascular catheter already in place, so will not cause their child any discomfort. If the infant does not have an indwelling catheter for blood draws, the parents and principal investigator will negotiate another plan. This plan will likely involve at least 2-3 blood draws at clinically indicated times so as not to stick the infant needlessly. Furthermore, they will be told that this amount of blood is not likely to contribute to the need for extra transfusions that may already occur as part of the routine care. Finally, they will be told that when their child's airway is suctioned, we will save the material for analysis instead of discarding it. Consent will be documented by the parent(s)' signature on a valid consent form approved by the Washington University Human Studies Committee.

The Washington University Human Studies Committee has not authorized a modification or waiver of the elements of consent or the requirements for documentation of consent.

*(4) Risks, alternatives*

The risks to the infant of participating in the study are minimal. The isotope infusions contain palmitate (bound to albumin), acetate, and dextrose, which are natural constituents of clinically used intravenous solutions for nutrition and intravascular volume expansion. The isotopes are naturally occurring and non-radioactive. The isotopes are prepared by a clinical pharmacist in a sterile fashion. There is a minimal risk of infection. This risk is no greater than the risk with any prepared intravenous fluid. The amount of blood required for the research aspects of the study is minimal in relation to that drawn for clinical care and should not contribute to the need for additional transfusions. The volume of fluid administered is less than 25% of maintenance for 24 hours only and can be factored into the overall fluid calculations. Electrolytes are measured regularly as part of routine intensive care monitoring. The remaining risks are those associated with being critically ill and requiring intensive care.

There are no alternatives to studying in vivo surfactant metabolism in humans.

*(5) Minimizing risks*

Current procedures in place in the intensive care units are designed to minimize the risks of intensive care procedures. Continuous review of infants' outcomes will occur according to a Data and Safety Monitoring Plan (DSMP) already in place. The data to be reviewed include: 1) certification of purity, sterility and pyrogenicity of isotope preparations, 2) number of subjects approached and number enrolled, 3) eligibility of subjects enrolled, 4) number of subjects for whom the study was not completed and reasons, 5) number of subjects who withdrew form the study and reasons, 6) number of subjects who developed electrolyte disturbances (acid-base balance, hypernatremia) during the isotope infusion, 7) subjects who die with in one month of the study, 8) subjects who develop bloodstream infections within 1 month of the study. The data will be reviewed every 3 months and compared to otherwise eligible infants who were not studied. These data will be provided to the Human Studies Committee annually. The Human Studies Committee will be notified in writing within 15 days of any adverse events that may be reasonable be regarded as being caused by, or probably caused by, the study protocol. Furthermore, if other complication, such as infection, electrolyte imbalance, etc, occur in excess of the rate in a comparable population of infants, the study will be stopped and the Human Studies Committee notified. Adverse events that might be anticipated will be reported if they are life threatening or result in death.

*(6) Risk benefit analysis*

As there are no alternatives to obtaining in vivo information about surfactant metabolism, and animal studies cannot mimic the pathophysiologic states being investigated, the information to be obtained from this proposal far outweighs the minimal risks associated with drawing an additional 2.5 ml of blood.

## 4) Literature Cited

1) Avery ME, Mead J. Surface properties in relation to atelectasis and hyaline membrane disease. Am J Dis Child 97:517-523, 1959.
2) Farrell PM, Avery ME. Hyaline membrane disease. Am Rev Resp Dis 111:657-688, 1975.
3) Martini WZ, Wolfe RR, Irtun O, Hawkins HK, Barrow RE. Dietary Fat Composition Alters Pulmonary Function in pigs. Nutrition 18 (numbers 7/8):647-653, 2002.
4) Bunt JE, Zimmermann LJ, Darcos-Wattimena JL, van Beek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. Am J Respir Crit Care Med 157: 810-814, 1998.
5) Cogo PE, Carnielli VP, Bunt JEH, Badon T, Giordano G, Zacchello F, Sauer PJJ, Zimmermann LJI. Endogenous surfactant metabolism in critically ill infants measured with stable isotope labeled fatty acids. Pediatr Res 45:242-246, 1999.
6) Martini WZ, Chinkes DL, Barrow RE, Murphey ED, Wolfe RR. Lung surfactant kinetics in conscious pigs. Am J Physiol 277:E187-195, 1999.
7) Patterson BW. Use of stable isotopically labeled tracers for studies of metabolic kinetics: An overview. Metabolism 46:322-329, 1997.
8) Batenburg JJ. Surfactant phospholipids: synthesis and storage. Am J Physiol 262:L367-85, 1992.
9) Jobe A. Ikegami M, Sarton-Miller I. The in vivo labeling with acetate and palmitate of lung phospholipids from developing a and adult rabbits. Biochimica et Biophysica Acta, 617:65-75, 1980.
10) Sato T, Akino T. Source of lung surfactant phospholipids: comparison of palmitate and acetate as precursors. Lipids 17(12):884-92, 1982 Dec.
11) Bohlin K, Merchak A, Patterson B, Hamvas A. Surfactant turnover is faster in infants with normal lungs compared to infants with lung disease. Pediatr Res 2001; 49:383A
12) Merchak A, Patterson BW, Yarasheski KE, Hamvas A. Use of stable isotope labeling technique and mass isotopomer distribution analysis of [(13)C]palmitate isolated from surfactant disaturated phospholipids to study surfactant in vivo kinetics in a premature infant. J Mass Spectrom. 2000;35: 734-8.
13) Martini WZ, Irtun O, Chinkes DL, Barrow RE, Wolfe RR. Surfactant phosphatidylcholine in thermally injured pigs. Crit Care Med. 2001;29:1417-22.
14) Bier DM. Stable isotopes in biosciences, their measurement and models for amino acid metabolism. Eur J Pediatr. 156:S2-8, 1997.
15) Garlick PJ, Cersosimo E. Techniques for assessing protein and glucose kinetics. Clin Endo Metab. 11:629-644 1997.
16) Bunt JEH, Carnielli VP, Seidner SR, Ikegami M, Wattimena JLD, Sauer PJJ, Jobe AH, Zimmermann LJI. Metabolism of endogenous surfactant in premature baboons and effect of prenatal corticosteroids. Am J Respir Crit Care Med 160:1481-1485, 1999.
17) Janssen DJMT, Carnielli VP, Cogo PE, Seidner SR, Luijendijk IH, Wattimena JLD, Jobe AH, Zimmermann LJI. Pool sizes and half lives of surfactant phosphatidylcholine in very premature baboons measured with stable isotopes. Am J Respir Crit Care Med 161:A568, 2000.
18) Merchak A, Jannsen D, Bohlin K, Patterson BW, Zimmermann LJ, Carnielli VP, Hamvas A. Endogenous pulmonary surfactant metabolism is not affected by mode of ventilation in premature infants with respiratory distress syndrome. J Pediatr 2002; 140:693-8.
19) Bohlin K, Merchak A, Spence, K, Patterson, B., Hamvas, A. Endogenous surfactant metabolism in newborn infants with and without respiratory failure. Ped Res.: submitted. See appendix.
20) Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium teteroxide. J Lipid Res. 1976;17:281-4.
21) Patterson BW, Zhao G, Elias N, Hachey DL, Klein S. Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment. J. Lipid Res 40:2118-2124, 1999.

## 5) Human Subjects/Vertebrate Animals

Vertebrate animals - none.

**30c. Respective Contributions**

Dr. Hamvas, my sponsor, has spent a great deal of time with me over the past year in the development and planning of this research proposal. Drs. Bruce Patterson and Aaron Hamvas have been accessible and very helpful in the analysis of preliminary data and in trouble-shooting daily issues that occur during research. The proposal was written by myself with extensive reviewing, idea-sharing and editing by myself and Drs. Hamvas and Patterson.

**30d. Selection of Sponsor and Institution**

I chose Dr. Hamvas to work with because his research subject, surfactant metabolism, and his methods are innovative and pertinent to neonatology, my subspecialty. Dr. Hamvas also performs translational medicine research. I plan to pursue a career in translational medical research as a physician scientist. He is also known for being accessible, enthusiastic, well-established and an expert in the surfactant field. Washington University is the only facility in the country using stable isotope methodology to study surfactant metabolism in human infants. Washington University also has an excellent clinical program in neonatology with a level III NICU. The NICU provides exposure to a high volume of patients with common as well as complex disorders and invaluable access to my study patient population. Such a large tertiary care and research center is the ideal location for a subspecialty fellowship and will provide many challenges to prepare me as a physician scientist.