A FORENSIC MARKER FOR A GENETIC DISEASE
OFTEN MISDIAGNOSED AS SUDDEN INFANT
DEATH SYNDROME (SIDS)

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Philip M. Kemp, B.S.

Denton, Texas

December, 1991
Kemp, Philip M., *A Forensic Marker for a Genetic Disease Often Misdiagnosed as Sudden Infant Death Syndrome (SIDS)*. Master of Science (Biomedical Sciences), December, 1991, 58 pp., 5 tables, 12 figures, bibliography, 36 titles.

Sudden Infant Death (SIDS) has been associated with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, an inborn error of fatty acid oxidation. Blood and tissue samples from a large cohort of SIDS victims were analyzed for the presence of dodecanoic acid (C_{12}) by gas chromatography. A subgroup of these cases had a significantly higher blood concentration than age-matched controls, suggesting MCAD deficiency.

An animal study using Sprague-Dawley rats was done to mimic the effects of MCAD deficiency. Significantly increased blood concentrations of dodecanoic acid were observed. Decreased values in heart and liver were puzzling findings.

The data indicate that dodecanoic acid is a blood marker for MCAD deficiency.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Methods and Materials</td>
<td>16</td>
</tr>
<tr>
<td>Human Studies</td>
<td></td>
</tr>
<tr>
<td>Animal Study I</td>
<td></td>
</tr>
<tr>
<td>Range Finding Studies</td>
<td></td>
</tr>
<tr>
<td>Animal Study II</td>
<td></td>
</tr>
<tr>
<td>III. Results</td>
<td>28</td>
</tr>
<tr>
<td>Human Studies</td>
<td></td>
</tr>
<tr>
<td>Animal Study I</td>
<td></td>
</tr>
<tr>
<td>Range Finding Studies</td>
<td></td>
</tr>
<tr>
<td>Animal Study II</td>
<td></td>
</tr>
<tr>
<td>IV. Discussion</td>
<td>41</td>
</tr>
<tr>
<td>Appendix</td>
<td>52</td>
</tr>
<tr>
<td>Reference List</td>
<td>55</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Urinary Acylglycine Excretion</td>
<td>14</td>
</tr>
<tr>
<td>2.1.</td>
<td>Dosage Groups for Animal Study I</td>
<td>23</td>
</tr>
<tr>
<td>3.1.</td>
<td>Dodecanoic Acid in Human Tissue</td>
<td>29</td>
</tr>
<tr>
<td>3.2.</td>
<td>Mean Concentrations of Dodecanoic Acid in Postmortem Blood</td>
<td>30</td>
</tr>
<tr>
<td>3.3.</td>
<td>Tissue concentrations of dodecanoic acid for the Three Animals in Range Finding Study #5</td>
<td>37</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Infant Mortality in King County Washington</td>
</tr>
<tr>
<td>1.2.</td>
<td>SIDS vs. Other Causes of Death</td>
</tr>
<tr>
<td>1.3.</td>
<td>Mobilization of Triglycerides</td>
</tr>
<tr>
<td>1.4.</td>
<td>Fatty Acid Membrane Transport and Oxidation</td>
</tr>
<tr>
<td>2.1.</td>
<td>Comparison of Dodecanoic Acid Retention Times</td>
</tr>
<tr>
<td>2.2.</td>
<td>Dodecanoic Acid From Standard Extraction</td>
</tr>
<tr>
<td>2.3.</td>
<td>Dodecanoic Acid Standard Curve</td>
</tr>
<tr>
<td>3.1.</td>
<td>Dodecanoic Acid in SIDS Victims</td>
</tr>
<tr>
<td>3.2.</td>
<td>Ratio of Dodecanoic Acid, Human Tissue:Blood</td>
</tr>
<tr>
<td>3.3.</td>
<td>Dodecanoic Acid in Blood of SIDS Victims</td>
</tr>
<tr>
<td>3.4.</td>
<td>Dodecanoic Acid, Rat Animal Model</td>
</tr>
<tr>
<td>3.5.</td>
<td>Ratio of Dodecanoic Acid, Rat Tissue:Blood</td>
</tr>
</tbody>
</table>
Sudden Infant Death (SIDS) is the modern name given to a 2,000 year old tragedy. It has been defined as the "sudden and unanticipated death in an infant, excluding the neonatal period, with no recognized lethal disorder" (Guntheroth 1989). This elusive entity has not been attributed to one specific cause but is presumed to be a heterogeneous group of disease processes (Di Maio and Di Maio 1989). One disease in this group involves a genetic defect in beta-oxidation, the cascade of reactions necessary for the conversion of fatty acids into energy (Stanley 1987). The absence of Medium Chain Acyl CoA Dehydrogenase, the catalyst for the initial reaction of beta-oxidation, has been associated with SIDS (Howat et al. 1985). Referred to as Medium Chain Acyl CoA Dehydrogenase (MCAD) Deficiency, this enzymatic defect may be responsible for a significant percentage of SIDS victims (Little et al. 1988). The purpose of this study is to delineate a marker for MCAD deficiency in the blood of a group of SIDS victims. By correlating it with established urinary markers, this fatty acid indicator in the blood may then be used to aid in the diagnosis of this metabolic disorder and in the identification of a distinct subgroup of SIDS victims.
The incidence of SIDS in proportion to live births in the United States ranges between two and three per thousand per year (0.2-0.3%). Relative to the number of live births each year, SIDS may be considered uncommon. In relation to infants dying after the first week of life, however, SIDS is the most common cause of death, comprising 40 to 60 percent of all deaths that occur after the first week of life and before the first birthday (Culbertson et al. 1981). In King County, Washington uniform diagnostic criteria were employed to study the occurrence of SIDS for the years 1969 - 1983. The data revealed that while other causes of infant death have declined through the years, SIDS has remained alarmingly constant, accounting for a growing proportion of total infant mortality (Fig. 1.1). Non-SIDS deaths decreased particularly during the latter half of the study period. This pattern can also be seen in studies from St. Louis, Missouri and Auckland, New Zealand.

The search is on for a common denominator, a connection linking all SIDS deaths together. Epidemiologic studies have shown that SIDS strikes blacks and Eskimos more often than whites. Infants born to young mothers, unmarried mothers, poor mothers, and uneducated mothers seem to be at a higher risk for SIDS. All of these factors are woven together but cannot be considered specific diagnostic markers for SIDS because they are also common components of other causes of infant mortality and survival.
Biochemical studies have yielded some interesting results, but none of the chemistries studied have been made specific markers for this problem. Levels of triiodothyronine (Chacon and Tildon 1981) and free thiamine (Davis, Icke, and Hitton 1982) were found to be elevated in SIDS babies when compared to babies dying of other causes. It has yet to be determined whether high concentrations of these two energy producing compounds are of any significance in identifying a baby at risk for SIDS. Guillian and colleagues suggested that perhaps the concentration of Hemoglobin F might be a marker for children at risk (Guillian, Gilbert and Moss 1987). Cortisol, growth hormone, and thyroid stimulating hormone have all been found to be in normal concentrations in the blood of SIDS children.
(Naeye et al. 1980). This same study revealed no differences in serum magnesium, phosphate, calcium, copper, and zinc values between SIDS victims and controls.

From the above data, the possibility arises that some genetic malfunction might play a role in sudden infant death. Cardiovascular function was approached by Maron and colleagues who suggested an autosomal dominant pattern of inheritance from their study of the QT interval (Maron et al. 1976). A study by Irgens et al shows that SIDS does occur in subsequent siblings (Irgens, Skjaerven and Peterson 1984). There is great difficulty, however, in estimating the probability of repetition in a family.

One area of genetic research that has received increasing interest is in the area of inherited metabolic disorders. It has been shown that MCAD deficiency is an autosomal recessive disease (Roe and Coates 1989). The present project focuses on fatty acid metabolism and a defect in the process of converting these organic acids into energy producing molecules (beta-oxidation). It has been suggested that inborn errors of fatty acid metabolism may occur in 1 out of 10,000 live births (Bennett, Worthy and Pollitt 1987). SIDS deaths occur at a rate of approximately 1 to 2 per 1,000 live births with some of these apparently having the medium chain acyl-CoA dehydrogenase deficiency (Howat et al. 1985). Death from MCAD is preventable if the deficiency is recognized,
therefore, it would appear that possibly 10% or more of these SIDS deaths would not have occurred had the defect been detected early. One of the goals of this study was to provide preliminary data for further investigation into the establishment of a rapid screening procedure whereby the serious consequences of MCAD deficiency may be prevented.

The age of the SIDS victim is a key element to understanding this syndrome and a possible link with inherited metabolic disorders. The only pattern of consistency among SIDS victims is the age of the child. SIDS rarely occurs in children less than one week of age or older than one year. The greatest number of SIDS victims appear to come from the one to four month range (Figure 1.2) with 80% of the deaths occurring before the age of 5 months (Goyco and Beckerman 1990).

Many important developments are taking place immunologically, morphologically, and metabolically during this time period. It is the age where many changes are taking place and the rate of growth is faster than it will ever be again. Changes in dietary intake are coinciding with the changes in intestinal flora. Sleep habits are just being established.

The severe nature of the symptoms of MCAD deficiency have been attributed to a decreased supply of energy for the brain from the lack of ketone bodies and to the toxicity of medium chain fatty acids (Duran et al. 1986).
An infant's brain makes significant use of ketones in order to meet the needs of its rapidly developing environment (Sokoloff 1989). One of the most important processes going on during this critical time period is the proliferation and integration of brain circuitry with the development of motor and sensory function. The ketone bodies produced by this oxidative process are an important source of energy necessary for proper function of the neuronal pathways. Fatty acids make up the mammalian body's major energy stockpile and are vital components of the lipids associated with cell membranes. They are stored as triacylglycerol in the form of adipose tissue. Having no other large pool of energy reserves, the body must call upon the fat stores to meet energy requirements after liver glycogen is depleted. After a 24 hour fast, the fatty acids
will provide as much as 80% of the adult caloric requirements, while during a prolonged fast, 94% of the energy needs will be met by fatty acids (Stanley 1987). An infant will show an even faster mobilization of fatty acids (between 12 and 24 hours) due to the energy demand of a proportionately large brain compared to body size. During prolonged fasting, the brain utilizes ketones derived from the oxidation of fatty acids in the liver to spare glucose.

Generally, during a period of fasting and/or exercise, the body perceives a need for more energy. Fatty acids are mobilized from the adipose tissue through a cascade of reactions initiated by epinephrine, norepinephrine, ACTH, or glucagon. These hormones produce cAMP upon binding with their receptors, and cAMP activates a protein kinase. The protein kinase then phosphorylates a hormone-sensitive lipase which can now remove a fatty acid from the stored triglyceride (Fig. 1.3). The free fatty acid leaves the adipose tissue and enters the circulation bound to albumin. Glycerol, the remaining entity following the removal of the three fatty acid moieties of the triglycerides, also enters the circulation to be oxidized in the glycolytic pathway via glycerol-3-phosphate.

Upon entering the cell, the fatty acid is converted to an acyl-CoA derivative by synthetase enzymes located on the outer mitochondrial membrane. The synthetases are specific with regard to chain length and the specificities overlap
such that virtually any saturated or unsaturated fatty acids, C₂-C₂₂, can be activated. The fatty acid acyl-CoA derivative can now cross the outer mitochondrial membrane but not the inner membrane. Carnitine acetyl transferase enzymes, also length specific, catalyze the binding of carnitine to the fatty acid for transport across the inner membrane (Fig. 1.4, a). Once inside the mitochondria, the fatty acyl-CoA derivatives are reformed and are now ready for beta-oxidation.

The initial reaction of beta-oxidation is the dehydrogenation of the fatty acyl-CoA catalyzed by specific dehydrogenase enzymes (c). Long chain acyl-CoA dehydrogenases act on fatty acids greater than twelve carbons in length, whereas, medium chain and short chain acyl-CoA dehydrogenases act on 6-12 and 4-6 carbon lengths respectively. Next, the double bond is hydrated, the catalyst being an enoyl hydratase (d), followed by cleavage of two carbons in a thiolase reaction (e). This process has now left a fatty acyl-CoA which has been shortened by two carbons and has produced an acetyl-CoA molecule which can now be oxidized in the tricarboxylic acid cycle or, in the liver, converted to ketones for further oxidation by other tissues such as the brain. The shortened acyl-CoA ester continues the oxidation process until it is completely converted to acetyl-CoA units. The initial dehydrogenase step (c), specifically, the medium chain acyl-CoA
Fig. 1.3. Mobilization of Triglycerides

\[
\begin{align*}
&\text{Triacylglycerol} \\
&\text{Glycerol} \\
&\text{Fatty Acids}
\end{align*}
\]

Fig. 1.4. Fatty Acid Membrane Transport and Oxidation

\[
\begin{align*}
&\text{Fatty acid} \\
&\text{Fatty acyl-CoA} \\
&\text{Carnitine} \\
&\text{Acetyl-carnitine} \\
&\text{Acyl-carnitine} \\
&\text{Carnitine} \\
&\text{Acyl-CoA} \\
&\text{FAD} \\
&\text{FADH}_2 \\
&\text{eBP} \\
&\text{Acyl-CoA (n-2)} \\
&\text{H}_2\text{O} \\
&\text{6-OH Acyl-CoA} \\
&\text{Acetyl-CoA} \\
&\text{8-Keto Acyl-CoA}
\end{align*}
\]
dehydrogenase, is the target of this research project. First described in 1982, MCAD is the most common of the inborn errors of fatty acid oxidation (Kolvraa et al. 1982). Victims of this disorder usually present in infancy with episodes of vomiting and lethargy that may progress to coma and death. These symptoms do not always appear before the first severe episode, however, making its clinical presentation unpredictable. The first episode usually occurs between 6 months and 2 years of age. The data gathered here would seem to indicate that these crises can indeed occur before the age 6 months.

Hypoketotic hypoglycemia is characteristic of the disease as is dicarboxylic aciduria. The defect in the oxidation process causes a fall in the production of ketones ("hypoketotic") and causes a large increase in the excretion of normal and abnormal urinary metabolites of fatty acids ("dicarboxylic aciduria"). Howat et al reviewed two hundred cases of sudden infant death to determine the proportion of cases with fatty changes of the liver with the intent of finding defects of fatty acid beta-oxidation (Howat et al. 1985). Fourteen of these cases had been described as having panlobular fatty changes in the liver. Five of these had frozen liver specimens available for histochemical studies. Two of the five had a defect in medium chain acyl-Coa dehydrogenase activity. Both of these cases were noted as having severe hypoglycemia, a finding
seen in victims of MCAD deficiency. Howat et al indicated that cases of sudden infant death with these fatty changes in the liver should be further investigated for defects in fatty acid metabolism.

Harpey and colleagues have suggested that MCAD deficiency has been misdiagnosed as Sudden Infant Death Syndrome (Harpey et al. 1987). They recommended that a screening procedure be established for the detection of the defect, especially for siblings of SIDS victims and those children who have experienced a "near miss" SIDS episode. Early detection could prevent the serious symptoms of MCAD deficiency from occurring or recurring.

Little et al associated MCAD deficiency with Sudden Infant Death Syndrome via the analysis of the blood in 250 SIDS victims by gas chromatography (Little et al. 1988). Twenty-one of these samples had a significantly higher concentration of dodecanoic acid (C₁₂ fatty acid) than did age matched control samples from non-SIDS causes of death (accident, disease, trauma). The implication here is that 8.4% of the samples from the SIDS group had a block or error in some phase of the fatty acid metabolism pathway. The lack of medium chain acyl-CoA dehydrogenase was proposed as the reason for excess amounts of dodecanoic acid.

MCAD deficiency is currently diagnosed by urinary organic acid profile, plasma carnitine levels, and enzymatic studies of liver, mononuclear leukocytes, and cultured
fibroblasts. The urinary organic acids, especially the identification of suberylglucose, provide a convenient means to diagnose MCAD deficiency. Stanley emphasized, however, that they are usually seen only in urine specimens obtained during fasting (Stanley 1987). The identification of octanoyl-carnitine, an excretion product of abnormal fatty acid oxidation, by fast atom bombardment-mass spectroscopy may also be used to diagnose MCAD (Roe et al. 1985).

When the normal pathway for fatty acid metabolism is not available, an alternate route is used to prevent the accumulation of the fatty acids in the blood. Biotransformation mechanisms must alter the molecules to facilitate elimination from the body. One of these mechanisms is conjugation, an enzyme catalyzed reaction involving the combination of a normally occurring substance and the target molecule. MCAD deficient subjects excrete large amounts of conjugates of medium chain fatty acids such as octanoylcarnitine and octanoylglucuronide (Duran et al. 1985). These conjugates occur as a result of the increased concentrations of medium chain fatty acids and their metabolites caused by the defect in beta-oxidation studied here. Glycine conjugates are also seen and have been demonstrated in patients who have trouble metabolizing short chain monocarboxylic acids (Ando et al. 1971). The identification of suberylglucose, an abnormal fatty acid-glycine conjugate, by gas chromatograph/mass spectrometry
has been suggested as a possible marker of MCAD in the urine (Gregerson, Gauritizen and Rasmussen 1976).

In addition, Rinaldo and colleagues have developed a procedure for the detection of acylglycines in the urine (Rinaldo et al. 1988). Their stable isotope dilution method is used for quantitating glycine conjugates of fatty acids in the urine. Suberylglycine, n-hexanoylglycine, and 3-propionylglycine are all identified by this method. The hexanoylglycine and the suberylglycine are abnormal by-products of fatty acid oxidation. The other metabolite, 3-phenylpropionylglycine, is formed by MCAD deficient subjects who are unable to metabolize phenylpropionic acid, which is a normal product of intestinal flora. The quantitation of this by-product may not be relevant, however, because the gut of the neonate does not always contain the intestinal flora necessary for its production. Suberylglycine was found to be of diagnostic use only in very high concentrations as normal infants also excrete this conjugate in the lower ranges. In Rinaldo's study all three of these abnormal metabolites showed significant increases above controls. Table 1.1 reflects the results of Rinaldo's study.

To validate the procedure, urine from samples with significantly high concentrations of dodecanoic acid were sent to Dr. Rinaldo's group at Yale University School of Medicine to be tested for MCAD deficiency using their stable
Table 1.1: Urinary Acylglycine Excretion
(ug/mg of creatinine)*

<table>
<thead>
<tr>
<th></th>
<th>MCAD DEFICIENT (n=16)</th>
<th>NORMAL (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanoylglycine</td>
<td>23.0 - 653</td>
<td>.21 - 1.9</td>
</tr>
<tr>
<td>Phenylpropionylglycine</td>
<td>2.5 - 180</td>
<td>0.0 - 1.1</td>
</tr>
<tr>
<td>Suberlyglycine</td>
<td>109.0 - 4553</td>
<td>0.0 - 95.0</td>
</tr>
</tbody>
</table>

* The student's t-test was used to compare values from each group; in all three cases p < 0.001.

isotope dilution method, an established diagnostic procedure for the detection of this disease.

An animal study was performed concurrently with the human research to further validate the screening procedure. Valproic acid (VPA) was employed in an attempt to mimic MCAD deficiency. Bjorge et al have demonstrated that VPA inhibits the beta-oxidation of the medium chain fatty acids in vitro (Bjorge and Baillie 1976). This property has also been demonstrated in vivo in Sprague-Dawley rats using VPA (Kesterson, Grauneman and Machinist 1984).

In the current study blood and tissue samples were taken from victims of Sudden Infant Death Syndrome as well as from infants dying of non-SIDS circumstances (homicide, disease, and trauma). The concentration of the dodecanoic
acid in SIDS victims were compared to that found in children that died of non-SIDS causes. These samples were quantitatively analyzed for the presence of dodecanoic acid by gas chromatography. During routine analysis of biological fluids from SIDS victims for the presence of acid and neutral drugs, we detected an increased concentration of a medium chain fatty acid. Identified as dodecanoic acid, this 12-carbon molecule was investigated to determine if it may serve as a blood marker for MCAD deficiency, a disease often misdiagnosed as sudden infant death syndrome.
METHODS AND MATERIALS

Human Studies

These studies were conducted with the approval of the Dallas County Institute of Forensic Sciences and the Dallas County Medical Examiner's Office. All analyses were performed in the toxicology laboratory of the Dallas County Institute of Forensic Sciences.

Blood samples were obtained from victims of Sudden Infant Death Syndrome (n=55) and from children dying from non-SIDS causes (n=50). Tissue samples of heart, liver, and muscle were also obtained when possible (SIDS, n=24; non-SIDS, n=7). The non-SIDS group served as controls. This group consisted of victims of trauma due to accident or homicide and those who succumbed to another identifiable disease. The age of all subjects was restricted to less than or equal to 1 year. Blood samples were stored in 10 mL. red top Vacutainer® test tubes. All blood samples were stored at 5°C until the analyses were performed. Tissue samples were kept at -71°C until time of analysis.

No special preparation was necessary for the blood samples before beginning the analysis. The tissue samples were prepared for analysis by homogenizing 10 grams in 30 milliliters (mL.) of deionized water. All reagents (AR
grade) were purchased from Fisher Scientific Company unless otherwise specified.

The samples were extracted using a procedure described in previous work (Foerster, Dempsey, and Garriott 1979). Five mL of blood or tissue homogenate were placed in a 15 mL screw cap culture tube. One hundred microliters (uL) of a 1 mg/mL solution of barbital (sodium salt in water) was added as an internal standard. A small amount (0.1 to 0.15 g) of potassium dihydrogen phosphate (KH₂PO₄) was added to adjust the pH to around 6. Five mL of toluene:diethylether (1:1, v:v) were added as the extracting solvent. This combination was allowed to mix for 5 minutes, followed by 5 minutes of centrifugation at 5000 rpm. The solvent (upper) layer was evaporated to residue by a stream of air without heating. The residue was reconstituted with 50 uL of acetonitrile (HPLC grade) and 500 uL of hexane (reagent grade). The extracts were mixed for 1 minute and centrifuged at 5000 rpm for 5 minutes. Three uL of the acetonitrile bead (bottom layer) were injected into the gas chromatograph.

The extracts were analyzed on a Hewlett-Packard 5890 gas chromatograph (GC) equipped with a Hewlett-Packard 3392A integrator to accumulate the data. The GC was fitted with a 5% phenylmethylsilicone capillary column (25m x .32mm x .52um). The analyses were carried out using a temperature program of 100° C to 290° C with a 10° C rise in temperature
per minute; the "initial" time was set at 0 minutes and the "final" time at 15 minutes. The injection port temperature was set at 250°C and the flame ionization detector temperature at 300°C. The samples were injected using a split injection technique which allowed 20% of the 1 uL sample to reach the column, the rest being carried away as waste. Helium (99.999% pure) was used as the carrier gas with the flow set at 40 cm/sec. The retention time of the compound was compared to that of a standard purchased from Sigma Chemical Co. (#L9755, Fig. 2.1). The final identification of the dodecanoic acid was done by computerized matching of the mass spectrum with that of the standard obtained from Sigma (Fig. 2.2). A 1 mg/L blood standard was extracted and injected on the GC/MS. A 99% match quality was observed when comparing the dodecanoic acid from Sigma and the peak seen in the SIDS autopsy blood.

A series of samples with increasing concentrations of dodecanoic acid were analyzed to validated the linearity of the procedure (Fig. 2.3). A stock standard (1 mg/mL) was prepared by dissolving 25 mg of dodecanoic acid in 25 mL of ethanol. The working standards for the linearity study were prepared by adding 5, 25, 50, 75, 100, 125, and 150 uL of the stock standard to 7 culture tubes. Five mL of blank blood were added to each tube producing a series of standards at 1, 5, 10, 15, 20, 25, and 30 mg/L. The working standards were then extracted according to the procedure
Fig. 2.1. Comparison of Dodecanoic Acid Retention Times

Fig. 2.2. Dodecanoic Acid From Standard Extraction on GC/MS
The quantitation factor for dodecanoic acid was obtained by dividing the peak area of the internal standard by the area of the dodecanoic acid and then multiplying this ratio by the concentration of the respective standard.

\[
\text{response factor} = \frac{\text{area of internal standard} \times \text{concentration}}{\text{area of dodecanoic acid}}
\]

The factor (amount/area) was determined to be 48.6 ± 2.04. When this factor is applied to Little et al's ratio for the 99th percentile in SIDS cases (Little et al. 1988), a concentration of 19.4 mg/L is obtained as the "cutoff" for preliminary diagnosis of MCAD deficiency.

The limits of detection and quantitation were determined by using peak areas. The width of the standard
peak was 1 mm (or 12 seconds of time). The area of the baseline for 12 seconds was 1043 units. Three times that area is defined as the limit of detection (LOD), while ten times the area is defined as the limit of quantitation (LOQ) (NIDA/CAP). Using a standard of known concentration, the detection and quantitation limits were determined by the equation on the following page. The limit of detection for dodecanoic acid using this methodology is 0.05 mg/L. The limit of quantitation is 0.16 mg/L.

\[ 3 \text{ or } 10 \times \text{area of baseline} \]

\[ \text{LOD or LOQ} = \frac{\text{area of standard}}{\text{area of baseline}} \]

To correlate the proposed screening procedure with published procedures for diagnosing MCAD deficiency, urine samples from 25 SIDS victims were sent to the Department of Human Genetics at the Yale University School of Medicine. Dr. Rinaldo's group analyzed them for the presence of abnormally high amounts of three fatty acid metabolites, n-hexanoylglycine, suberylglycine, and 3-phenylpropionylglycine. The procedure is a stable isotope dilution method involving the addition labelled acylglycines to the urine samples and extracting them according to their previously published method (Rinaldo et al. 1988).

One urine sample was analyzed at the Department of Pediatrics of Duke University Medical Center. The procedure
is a urinary organic acid and acylcarnitine profile done by GC/MS (Millington et al. 1990). The liver tissue was also analyzed for the presence of the genetic abnormality associated with MCAD deficiency (Matsubara et al. 1990).

**Animal Studies**

Two animal studies were performed concurrently with the human experimentation. The first was an attempt to substantiate the hypothesis that some sudden infant deaths may be associated with a genetic defect in fatty acid metabolism, Medium Chain Acyl-CoA Dehydrogenase Deficiency (MCAD). The second study was an attempt to show that the blockage of the beta-oxidation of fatty acids resulted in elevated concentrations of dodecanoic acid in the blood and tissues. Sprague-Dawley rats were used in both studies. Valproic acid (VPA) was employed in each experiment to block the metabolism of fatty acids and create a condition similar to MCAD.

**Animal Study I**

The first experiment was an attempt to test the hypothesis that the blockage of the beta-oxidation process caused an increase in the concentration of dodecanoic acid resulting in a build-up of fatty acids causing sudden death as seen in SIDS victims. The procedure was begun with 27 day old animals weighing an average of 65 g. Twenty-seven days was the time necessary for weaning, a requirement set
down by the IRB reviewing the procedure. The rats were divided into 5 groups according to the dosages to be administered (table 2.1). The values for the LD5 (13.1 mg/kg), LD50 (131 mg/kg), and LD90 (236 mg/kg) were calculated using previously published data for the LD50 of dodecanoic acid (Oro and Wretlund 1961).

All of the group, except the controls, were given a single dose of 300 mg/kg VPA, by gavage, for 3 days prior to beginning the above dosage protocols to initiate the inhibition of fatty acid metabolism.

Table 2.1 Dosage groups for Animal Study I

1. 300 mg/kg VPA
2. 300 mg/kg VPA + LD5 dodecanoic acid
3. 300 mg/kg VPA + LD50 dodecanoic acid
4. LD90 dodecanoic acid
5. Control group

The animals in each group were then given a single dose each day, by gavage, of their respective mixtures seen in table 2.1 for an eleven day period. All groups were fasted for 12 hours following the first and second dose. The animals were put on a 36 hour fast beginning with dose 9 and continuing through doses 10 and 11.

After 11 days of this study, none of the desired results occurred. Smaller experiments ("Range Finding
Studies") were then done in an attempt to determine if the
dodecanoic acid was being lost by some unknown mechanism or
if a much larger dose was needed to achieve death when
administering the acid by gavage. The animals used for
these smaller tests were the same rats used in Animal Study
I, therefore, the weights and ages were increased
accordingly.

Range Finding Studies (Animal Study I)

1. Two groups of three rats each (39 days old, 121 g)
were given increased dosages of dodecanoic acid. The first
group was given 500 mg/kg (approx. 2 x LD90) and the second
1000 mg/kg (approx. 4 x LD90) by gastric intubation. These
animals were fasted for 12 hours and observed for any signs
of stress like that which is seen in MCAD deficiency. The
question to be answered here was whether or not we could
induce sudden death by going well above the calculated LD90
of the dodecanoic acid.

2. One of the animals in group 3 of Animal Study I
died on the 10th day as a result of misplacing the gavage
tube in the trachea. The animal was autopsied and the liver
was removed to be analyzed for the presence of dodecanoic
acid. The purpose of this autopsy was to see if the
dodecanoic acid was reaching the liver or was being lost by
some unknown mechanism.

3. Two groups of three rats (42 days old, 140 g) were
given 600 mg/kg VPA and 600 mg/kg VPA + 1000 mg/kg
dodecanoic acid respectively. The animals were then fasted for 12 hours and observed for any signs of stress or morbidity. This experiment added VPA for the inhibition of beta-oxidation to see if the combination of the large dose of dodecanoic acid and VPA could cause death or stress.

4. One rat (60 days old, 146 g) was given 600 mg/kg VPA + 236 mg/kg dodecanoic acid (LD90). The animal was placed in a clean cage for 24 hours and fasted for that length of time. The feces was collected after 24 hours and analyzed for dodecanoic acid; the purpose was to determine if the dodecanoic acid was being eliminated in the feces without being absorbed.

5. Three rats (62 days old, 135 g) were separated and two were dosed with 600 mg/kg VPA (by gavage). The third (control) was given water only. The animals were fasted for 24 hours at which time the first was given VPA again, the second was given the VPA + LD90 dodecanoic acid, and third was given water. The animals were sacrificed and the heart and liver taken for analysis of the dodecanoic acid concentration.

6. Three rats (69 days old, 336 g) were administered the LD90 dodecanoic acid i.v. (tail vein). These animals were observed for a period of 8 hours for sudden death or any signs of morbidity.

Animal Study II

The second animal study was performed in an attempt to
show that when the metabolism of fatty acids is interrupted, the concentration of dodecanoic acid increases. This would validate the proposed screening procedure and verify dodecanoic acid as a marker for MCAD deficiency in the blood.

Valproic acid was given to Sprague-Dawley rats averaging 200 g in weight. The animals were administered 600 mg/kg VPA in water by gastric intubation. They were given the normal amounts of food and water over the course of the experiment.

Twenty-five control animals (no drug administered) were sacrificed. Blood and tissue samples (heart and liver) were taken immediately and stored until time of analysis. The blood was refrigerated at 5°C and the tissues were kept frozen at -71°C.

Twenty-five animals were used for the VPA study. All animals were dosed with the 600 mg/kg VPA on the first day. On the second day, 5 animals were sacrificed and blood, heart, and liver samples taken. The 20 remaining animals were again dosed with VPA. Each day thereafter, 5 animals were sacrificed and the remaining subjects were dosed with another 600 mg/kg VPA. This procedure was continued until all animals were sacrificed, a total of 5 days.

The blood samples were extracted and analyzed according to the protocol given in the human study. The tissue samples were homogenized in deionized water in a 1:10
dilution for heart and 1:4 dilution for the liver. These homogenates were extracted and analyzed according to the aforementioned procedure.
CHAPTER 3

RESULTS

The first objective of this study was to determine if dodecanoic acid concentrations in the postmortem blood and tissues of all SIDS victims were significantly different from the controls. A significant difference in these concentrations may point to a metabolic disorder common to SIDS cases. The results of these blood and tissue analyses are shown in figure 3.1. The mean blood concentration for all SIDS children (7.65 ± 0.70 mg/L) was not significantly different from the controls (6.46 ± 0.84 mg/L), indicating that an elevated quantity of dodecanoic acid in the blood is not a marker for SIDS itself. This was not entirely surprising due to the fact that SIDS, quite probably, has a heterogeneous etiology.

The mean concentrations of dodecanoic acid in the heart, liver, and muscle are shown in table 3.1 for those cases in which these samples were available. The control tissue data is comprised of children who were the victim of an identifiable disease (n=7). Disease victims were the only non-SIDS cases in which the tissues could be obtained. Accident and trauma victims are relatively rare for this age group and even then, the tissues may not be available for study.
Table 3.1 Dodecanoic acid in human tissue (mg/kg).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Heart</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS</td>
<td>24</td>
<td>16.0± 2.3</td>
<td>24.4± 3.7</td>
<td>20.5± 3.6</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>7</td>
<td>43.6±17.6</td>
<td>34.8±16.4</td>
<td>53.7±35.4</td>
</tr>
</tbody>
</table>

Figure 3.2 shows the human dodecanoic acid data as a comparison of the ratio of the blood value to that of the tissues. This was done in an attempt to determine if the ratio of blood concentration to tissue concentration could be an important relationship to consider when looking for MCAD deficiency in SIDS children. In forensic toxicology, the tissue concentrations of drugs are important factors to consider when trying to verify intoxication or overdose situations by comparing them with the blood values. The thought here was to obtain a similar comparison for dodecanoic acid through this mathematical relationship.

The focus of the study then turned to the primary objective of this work, whether or not dodecanoic acid is a marker in blood for MCAD deficiency. When the SIDS group was isolated and analyzed for the presence of dodecanoic acid, three cases out of 55 had an elevated (>19.4 mg/L) concentration in postmortem blood. The mean concentrations of dodecanoic acid in these cases as well as for the other SIDS cases and control groups are shown in table 3.2 and in
Table 3.2 Mean Concentrations of Dodecanoic Acid in Postmortem Blood (mg/L).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAD Deficient</td>
<td>3</td>
<td>22.6 ± 2.2</td>
</tr>
<tr>
<td>SIDS (not MCAD)</td>
<td>52</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>Trauma</td>
<td>31</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>Disease</td>
<td>13</td>
<td>5.4 ± 1.5</td>
</tr>
<tr>
<td>Undetermined*</td>
<td>6</td>
<td>6.8 ± 2.0</td>
</tr>
</tbody>
</table>

The MCAD group was significantly different from all of the control groups (p<0.001).
* Those cases where violence or disease are strongly suspected but cannot be validated.

The three suspected MCAD deficiency cases had to be confirmed by proven methodology. Twenty-five urine samples, including two of the three cases with elevated concentrations of dodecanoic acid (mean = 22.6 ± 2.2 mg/L) were sent to the Department of Human Genetics at Yale University for the identification of abnormal fatty acid oxidation metabolites using Rinaldo's stable isotope dilution technique. The third sample with elevated
Dodecanoic acid in SIDS Victims

mg/L for blood or mg/Kg for tissue
Fig. 3.2

Ratio of Dodecanoic Acid
Human Tissue:Blood

![Bar chart showing the ratio of dodecanoic acid in SIDS and Disease Controls for different tissues compared to blood.](image)

- Liver:Blood
- Heart:Blood
- Muscle:Blood

mg/Kg:mg/L
Dodecanoic Acid (mg/L) in Blood of SIDS Victims

![Bar Chart]

- MCAD Deficiency
- SIDS
- Disease
- Trauma
- Undetermined
Dodecanoic acid was sent to Duke University by the pathologist working the case to verify his suspicion of a metabolic disorder. All three of the suspected cases were diagnosed as having MCAD deficiency. None of the control (without the high dodecanoic acid) were positive for the disease. These findings resulted in a high degree of confidence for dodecanoic acid being a marker for MCAD deficiency (p<0.0005).

Animal Studies

Animal Study I

Past research had shown that valproic acid will block the beta-oxidation of fatty acids. Valproic acid was chosen, therefore, to mimic MCAD deficiency in 27 day old Sprague-Dawley rats. Fasting periods were used to initiate the mobilization of fatty acids to be used as sources of energy. LD 5, 50, and 90 doses of dodecanoic acid were administered to test the toxicity of this particular fatty acid under MCAD-like conditions. It was expected that symptoms resembling those seen in MCAD deficient children, even to the point of sudden death, would be seen in a significant percentage of the animals.

However, none of the expected results were seen in the 11 day study. Even a 36 hour fasting period combined with dodecanoic acid and valproic acid doses could not produce visible stress of any kind. The i.v. administration of LD90 dodecanoic acid again proved to be ineffective in producing
visible MCAD-like symptoms. It was decided to pursue the reason for these unexpected results with a series of "range finding studies". Experiments were designed to find the range of doses for the VPA-dodecanoic acid combination that would produce the same type of symptoms and/or sudden fatal episode associated with MCAD deficiency.

Range Finding Studies

The range finding studies that were done as a result of Animal Study I yielded some interesting data. The data is presented in the same order as found in chapter 2.

1. Two rats were given dodecanoic acid concentrations, by gavage, approximately 2 and 4 times the LD50 administered i.v. in previous research. No deaths were observed and no signs of stress resulted from these doses even after 12 hours of fasting to initiate the beta-oxidation process.

2. The analysis of the liver of a rat which died on the 10th day of Animal Study I revealed that this organ was accumulating large amounts of dodecanoic acid. Death was due to misplacement of the gavage tube in the trachea. This animal had gone through the fasting periods, dosages of VPA, and high doses of dodecanoic acid for a period of 10 days. It is unclear which of these variables contributed the most extensively to the large amount of the dodecanoic acid found in the liver (150 mg/kg). This concentration was a much higher value than was found in any of the liver samples, control or "MCAD", that would later be seen in Animal Study
II. It was also higher than most of the human liver tissue analyzed, and yet, this animal showed no outward signs of the effects of the artificial MCAD deficiency.

3. The six rats in this experiment showed no signs of stress and no sudden death resulting from the large doses of VPA and dodecanoic acid administered. The 12 hour fast should have again initiated the beta-oxidation mechanism which theoretically should have produced MCAD-like symptoms.

4. The following concentrations of dodecanoic acid were found in the feces of the control and the experimental rat after a 24 hour study in which one of the animals was given large doses of VPA and dodecanoic acid.

\[
\begin{align*}
\text{control} &= 0.02 \text{ mg} \\
\text{experimental} &= 0.12 \text{ mg (0.35\% of dose)}
\end{align*}
\]

These data would seem to indicate that the dodecanoic acid was not being eliminated to a significant degree via the feces.

5. The heart and liver of the 3 animals in this experiment were analyzed for the presence dodecanoic acid. The results are presented in table 3.3. It is important to note that the tissue concentrations of the C\textsubscript{12} fatty acid are elevated above the controls for both the "VPA only" and the combination doses. This would seem to validate the hypothesis that MCAD deficiency produces an increased
concentration of dodecanoic acid and that this change is detectable by flame-ionization gas chromatography.

Table 3.3 Tissue Concentrations of Dodecanoic Acid for the Three Animals in Range Finding Study #5 (mg/kg)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Heart</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>5.3</td>
<td>1.1</td>
</tr>
<tr>
<td>2. VPA only</td>
<td>19.4</td>
<td>22.2</td>
</tr>
<tr>
<td>3. VPA + Dodecanoic Acid</td>
<td>32.6</td>
<td>17.3</td>
</tr>
</tbody>
</table>

6. Even after i.v. administration of the calculated LD90 dodecanoic acid, the animals in this procedure demonstrated no signs of stress. They were observed for a period of 8 hours without any outward signs of discomfort.

It became clear that the symptoms of MCAD deficiency are difficult to reproduce in the laboratory in an animal model.

Animal Study 11

The concentrations of dodecanoic acid found in this study are presented in figure 3.4. It is clear that the VPA did cause a significant increase ($p<0.005$) in the blood concentration by day 2, presumably through the blockade of beta-oxidation. This implies that MCAD deficiency causes
increased quantities of the $C_{12}$ fatty acid in the circulation. It is interesting to note that the concentrations fall after day 2 and continue to fall through day 5. Day 5 is significantly small ($p<0.02$) compared to day 2. The blood and liver concentrations for day 5 actually fell below the calculated limit of detection but the data was shown for the sake of completeness.

The steady decline in concentration of dodecanoic acid after Day 2 would seem to indicate that a mechanism other than beta-oxidation was able to adapt, or increase metabolism, of the fatty acid and allow the elimination processes to remove the dodecanoic acid from the blood.

As with the human study, the comparison of blood and tissue ratios are shown in figure 3.5. The ratios of tissue:blood decrease precipitously from the controls on day 1 with an upward trend beginning on the third day. The pattern again indicates the increasing blood concentrations up to day 2 as well as showing the declining tissue concentrations as they decrease from the controls.
Dodecanoic acid
Rat Animal Model

Fig. 3.4

mg/L for blood or mg/Kg for tissue
Fig. 3.5

Ratio of Dodecanoic Acid
Rat Tissue:Blood

Liver:Blood

Heart:Blood

mg/Kg:mg/L
CHAPTER 4

DISCUSSION

MCAD deficiency has been a specialized diagnosis, often relying on postmortem tissue assays, cultured fibroblast and leukocyte studies (Coates et al. 1985), or a urinary organic acid profile (Pollitt 1987). These studies are certainly valuable, but not widely available, and have the added disadvantage of being time-consuming and costly. In addition, urine samples are seldom available for postmortem examination in these children because of micturation at the time of death.

The primary objective of this project was to determine if a high concentration of dodecanoic acid, seen in the postmortem blood of children thought to be victims of Sudden Infant Death Syndrome, could actually be a marker for MCAD deficiency. If so, this procedure could be incorporated into many forensic laboratories that use gas chromatography as a drug screening tool.

The data strongly suggest that, indeed, dodecanoic acid is a valid predictor in postmortem blood for MCAD deficiency (Fig. 3.3). Concentrations greater than 19.4 mg/L may be indicative of the MCAD variant of the SIDS death. Often times these compounds are labelled as simply "normal autopsy blood components" or "contaminants" when in fact, they may
be an indication of this inherited metabolic disorder. It is important to note from this figure that the mean dodecanoic acid concentrations found in the MCAD deficient children are significantly higher than even the non-MCAD, diseased controls. Most of the diseases were respiratory in nature, which are conditions known to elevate blood fatty acid concentrations. Positional asphyxia also causes an increase in fatty acid concentration but not to the level seen in MCAD victims. The forensic toxicologist should then, in most cases, be able to make a preliminary identification of this beta-oxidation disorder.

Two of the MCAD deficiency cases in the present study were confirmed by the stable isotope dilution method of Rinaldo et al. The third case was confirmed at Duke University by Roe's group using a combination of urinary organic acid profile by GC/MS and a tandem mass spectrometry method for the analysis of urinary acylcarnitines. The fact that the elevated blood dodecanoic acid correlated well with two outside laboratory diagnoses argues favorably for elevated concentrations of dodecanoic acid ($C_{12}$) fatty acid being a blood marker for MCAD deficiency.

The finding of excess dodecanoic acid in 3/55 (5.4%) of SIDS children also correlates well with the early data from Little et al (21/250; 8.4%) (Little et al. 1988) and from a recent publication by Harpey and colleagues in which they mention an elevated level of this fatty acid in the blood of
10 siblings of SIDS and 4 "near miss" infants. (14/273; 5.1%) (Harpey, Charpentier and Paturneau-Jouas). They go on to state that normal newborn infants have blood C12 values of 2-5 mg/L and that older infants have values of 1-3 mg/L. The mean values for controls analyzed in the current study were slightly higher than these data (6.85 mg/L) but this can possibly be explained by postmortem changes, whereas, Harpey's specimens were antemortem. The fact that Harpey's samples were from siblings of SIDS cases suggests that there is a need for testing the families of children found to have a confirmed diagnosis of MCAD deficiency. The estimated recurrence rate of fatty acid beta-oxidation disorders is 25% due to autosomal recessive transmission.

Animal Study I was not successful, it appeared, in providing much information regarding MCAD deficiency and its relationship to Sudden Infant Death Syndrome. However, there were some interesting findings. The dodecanoic acid is not acutely toxic as demonstrated by the lack of untoward effects resulting from extremely high doses by gavage and i.v. administration of LD90 dodecanoic acid. This finding contradicts previous research (Oro and Wretlund 1961).

The valproic acid was effective in blocking the beta-oxidation process as evidenced by range finding study #2 in which the analysis of the liver of an animal that died from unrelated causes revealed a concentration of dodecanoic acid well above the mean liver concentrations found in other
experiments. This effect was also evident in range finding study #5. In this experiment the heart and liver $C_{12}$ values found in 2 rats administered VPA were elevated to a concentration much higher than the control animal.

One of the problems encountered in Animal Study I was the insolubility of high concentrations of the dodecanoic acid in water. Even the sodium salt was difficult to dissolve at the higher concentrations. This problem was alleviated by the use of a small amount of heat to dissolve the fatty acid. This was an important dilemma to solve because of the desire to administer the valproic acid and dodecanoic acid in the same dosing solution to avoid a second gavage.

In addition, Animal Study I did not produce the expected number sudden deaths resulting from the "MCAD-like" condition imposed on the animals. These animals survived despite 11 days of valproic acid + dodecanoic acid and intermittent fasting after each dose. This may suggest that the genetic material responsible for the disease must be present to have the fatal effect and valproic acid may not be able to effectively mimic the specific symptoms of this disease. The enzymes may have to develop abnormally from the beginning rather than be inhibited artificially to produce MCAD deficiency symptoms.

The findings in Animal Study II would seem to validate the technique for using gas chromatography for the
identification and quantitation of dodecanolic acid in postmortem blood, and a preliminary diagnosis of MCAD deficiency. Upon imposing the "MCAD-like" condition on the Sprague-Dawley rats with valproic acid, the $C_{12}$ concentrations increased rapidly to a significantly elevated value within 48 hours (Fig. 3.4). The beta-oxidation blockade appeared to have been overridden by an unknown mechanism(s) and the concentrations began to decrease by Day 3. Possible explanations for this "adaptation" may come from the alternate pathways for the elimination of excess fatty acids, conjugation with carnitine and/or glycine and omega-oxidation. It would not be surprising for conjugation metabolites to go undetected on GC due to their increased water solubility. Urine from the animals should be investigated for greatly increased quantities of these metabolites via more specific techniques such as hydrolysis followed by derivatization.

The results of the tissue data are not clear regarding the possibility of an MCAD diagnosis from analysis of these specimens. These data may, however, provide information for further investigation into the etiology of SIDS. The data shown in Fig. 3.1 indicate a smaller tissue concentration of dodecanolic acid in SIDS infants as compared to controls. The pattern seen in the human data in Fig. 3.1 and that for the animal data in Fig. 3.4, up to Day 2, are very similar. The SIDS group liver, heart, and muscle are lower in
concentration than the controls. The most MCAD-like group in Day 2 shows the same pattern, even to the point that the liver concentration slightly surpasses that of the heart, a switch from the controls. An interesting note here is that 63% of the SIDS cases analyzed for tissue concentration of C₁₂ showed this pattern of the liver and heart values being reversed (liver > heart) from the normal subjects. Whether or not this has any relevance to the etiology of SIDS has yet to be determined but should be investigated.

Another observation to be made from the animal data in Fig. 3.4 is the steady decline in tissue concentration (both heart and liver) from the control data. Day 1 and 2 show increasing blood values as the tissue concentrations fall. After Day 2 the blood levels decline and the tissue levels continue to diminish. It appears that the dodecanoic acid is leaving the tissues, being metabolized via conjugation, etc., and then being eliminated. The dodecanoic acid is apparently not being excreted unchanged in the feces, a fact revealed in Animal Study I by the 24 hour fecal experiment performed during the range finding study and discussed in the preceding chapter. Why is the dodecanoic acid in SIDS victims apparently lower than the controls? Why do the blood levels decline after the second day of Animal Study II? These are questions needing further investigation.

A problem with current SIDS research is the accumulation of proper control data. A potential criticism
of the present study may be that the control tissue data seen in figure 3.1 may have been skewed by the fact that all of these specimens were from children dying as a result of an identifiable disease (other than MCAD), the majority being respiratory in nature. These subjects could conceivably have had elevated blood and tissue levels of dodecanoic acid due to their illness. No tissue specimens were available for the trauma control group which would provide the most "normal" concentrations of dodecanoic acid (these cases are difficult to obtain, but are the best samples for controls available). As a result, the comparison of tissue concentrations among the control groups cannot be made. This argument does not hold true for the blood, however, as seen in figure 3.3. There is virtually no difference in the blood concentrations found in any of the control categories, including the SIDS cases not diagnosed with MCAD.

It is unfortunate that no tissues were available from the MCAD-confirmed children. This would have been valuable for comparison to the control and SIDS tissue data. The tissue distribution of the dodecanoic acid in these children should be investigated to determine if these data could be useful for the study of MCAD and for determining the cause of death in this age group.

The ratio of tissue:blood was calculated in both human and animal studies (Fig. 3.2 and 3.5) to determine if this
relationship might be important in diagnosing a death resulting from MCAD deficiency and to see if it has relevance to the SIDS phenomena itself. The ratio of liver:blood and heart:blood is lower for the SIDS population than the controls. The same can be said for the dosage group data in Animal Study II. This could indicate a common mechanism for the processing of fatty acids in animals with the "MCAD-like" condition and the SIDS children. From the human data in Fig. 3.1, it would appear that the blood concentration of dodecanoic acid is not affected by the compensatory mechanisms in MCAD cases. The tissue value, therefore, must have significantly decreased to produce the ratios observed.

An interesting observation can be made from the tissue data in SIDS children. That is, it may be an indication of the extent of suffering that these children endure just before death. Lachica et al. concluded that an extended period of agonal suffering, as seen in prolonged illnesses, causes a significant increase in the fatty acid content of the heart (Lachica, Villanueva and Luna 1988). The mean concentration of dodecanoic acid seen in the heart of SIDS children was less than the controls. These data then would seem to confirm the long held belief that these children do not suffer for any prolonged period of time, that their death is usually quite sudden.

From these experiments, it is apparent that dodecanoic
acid is not the causitive agent in the sudden death of these MCAD infants. This hypothesis was confirmed in the range finding study in which the i.v. administration of the LD90 dose did not cause any untoward effects much less death. The accumulation of toxic quantities of acyl-CoA's in the mitochondria and/or the lack of ketones necessary for energy have been considered the reasons for the failure of these children to survive (Duran et al. 1986).

Dodecanoic acid has also been shown to promote hemolysis of red blood cells in a hypotonic environment when present in a large enough concentration (Rybczynska and Csordas 1989). The fatty acid intercalates into the cell membrane, thus, disrupting the fragile organization of the lipid bilayer, a detergent effect. The current study indicates that this phenomena does not have a lethal effect acutely (i.v. administration of LD90 during the range finding experiments). Over a prolonged period of time, however, this effect may be a contributory factor to death associated with MCAD deficiency. It may be that if this effect is observed in MCAD victims, it is the result of the accumulation of large quantities of medium chain fatty acids, in addition to dodecanoic acid.

Carroll and colleagues have reported that fatty acid oxidation rates tend to increase with age in developing heart, muscle, and liver of the rat (Carroll et al. 1989). They analyzed these three tissues for acyl-CoA dehydrogenase
activity from birth to adulthood in Sprague-Dawley rats. Medium chain acyl-CoA dehydrogenase reached near-adult levels by 11 days of age. Unfortunately, we were not allowed to use the young rats until at least 21 days of age (weaning age) as required by the Institutional Review Board. It would be of interest to be able to observe the effect of blocking beta-oxidation on the pregnant female and the neonate rat as well as the effect of the additional dodecanoic acid.

If such an immaturity exists in some human infants immediately after birth, these children could be at risk for a potentially fatal episode as seen in MCAD victims. It would be beneficial to have both a urine and a blood screening procedure for newborns for the detection of MCAD deficient infants, who actually have the genetic disease, and for those children having the immature enzyme system. Therefore, the blood marker dodecanoic acid, may be useful in the development of such a blood screen.

Toxicology studies are an integral part of the medicolegal investigation of the sudden death of a child at the Dallas County Institute of Forensic Sciences. More often than not, these studies are negative. The possibility now exists, however, that the gas chromatographic screen for acid and neutral drugs may provide an important clue in a certain percentage of these difficult cases. Dodecanoic acid is easily detectable in postmortem blood in the range
given in this study as diagnostic for MCAD deficiency (>19.4 mg/L). Parents of such a child may then be referred for genetic testing and counseling and siblings may be tested for the disease to prevent the possible lethal effects.

The proposed screening procedure should be investigated for use in the clinical setting as well, since the possibility exists that a proportion of SIDS deaths may be preventable. MCAD deficiency is treatable by avoiding the need to metabolize fatty acids. Carbohydrate rich meals and carnitine supplementation are two treatments which have been successful. The administration of riboflavin could increase acyl-CoA dehydrogenase activity. Prolonged fasting periods should also be avoided.
DATA USED TO CONSTRUCT FIGURES

**Fig. 3.1** Dodecanoic Acid in SIDS Victims (Blood, mg/L; Tissue, mg/kg)

<table>
<thead>
<tr>
<th>SIDS</th>
<th>N</th>
<th>Mean ± SEM</th>
<th>Controls</th>
<th>N</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>55</td>
<td>7.65 ± 0.70</td>
<td>50</td>
<td>50</td>
<td>6.46 ± 0.84</td>
</tr>
<tr>
<td>Liver</td>
<td>24</td>
<td>24.4 ± 3.70</td>
<td>7</td>
<td>7</td>
<td>34.8 ± 16.4</td>
</tr>
<tr>
<td>Heart</td>
<td>24</td>
<td>16.0 ± 2.30</td>
<td>7</td>
<td>7</td>
<td>43.6 ± 17.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>24</td>
<td>20.5 ± 3.60</td>
<td>7</td>
<td>7</td>
<td>53.7 ± 35.4</td>
</tr>
</tbody>
</table>

**Fig. 3.2** Ratio of Dodecanoic Acid (Human tissue: Blood)

<table>
<thead>
<tr>
<th>SIDS</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver:Blood</td>
<td>2.99</td>
</tr>
<tr>
<td>Heart:Blood</td>
<td>2.28</td>
</tr>
<tr>
<td>Muscle:Blood</td>
<td>2.71</td>
</tr>
</tbody>
</table>

**Fig. 3.3** Dodecanoic Acid (mg/L ± SEM) in Blood of SIDS Victims

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAD Deficient</td>
<td>3</td>
<td>22.6 ± 2.2</td>
</tr>
<tr>
<td>SIDS (not MCAD)</td>
<td>52</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>Trauma</td>
<td>31</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>Disease</td>
<td>13</td>
<td>5.4 ± 1.5</td>
</tr>
<tr>
<td>Undetermined</td>
<td>6</td>
<td>6.8 ± 2.0</td>
</tr>
</tbody>
</table>

**Fig. 3.4** Dodecanoic Acid - Rat Animal Model (Blood, mg/L; Tissue, mg/kg; ± SEM)

<table>
<thead>
<tr>
<th>N</th>
<th>Blood</th>
<th>Liver</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.14 ± 0.02</td>
<td>0.95 ± 0.24</td>
<td>22.0 ± 3.0</td>
</tr>
<tr>
<td>5</td>
<td>0.37 ± 0.13</td>
<td>0.64 ± 0.32</td>
<td>8.8 ± 1.8</td>
</tr>
<tr>
<td>5</td>
<td>1.59 ± 1.45</td>
<td>0.52 ± 0.25</td>
<td>5.9 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td>0.43 ± 0.12</td>
<td>0.40 ± 0.11</td>
<td>9.2 ± 2.1</td>
</tr>
<tr>
<td>5</td>
<td>0.38 ± 0.03</td>
<td>0.24 ± 0.09</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.05</td>
<td>3.8 ± 0.2</td>
</tr>
</tbody>
</table>
Fig. 3.5 Ratio of Dodecanoic Acid  
(Rat Tissue:Blood)

<table>
<thead>
<tr>
<th></th>
<th>Liver:Blood</th>
<th>Heart:Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9</td>
<td>157.0</td>
</tr>
<tr>
<td>Day 1</td>
<td>1.0</td>
<td>24.1</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.9</td>
<td>21.1</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.6</td>
<td>18.4</td>
</tr>
<tr>
<td>Day 5</td>
<td>1.0</td>
<td>61.6</td>
</tr>
</tbody>
</table>
REFERENCE LIST


NIDA/CAP recommendations for calculating LOD and LOQ.


