THE EFFECT OF EARLY FEEDING ON HUMAN CHOLESTEROL SYNTHESIS AT FOUR MONTHS OF AGE

by

Janet L. Bulani
B. Sc., University of Saskatchewan, 1989

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(School of Family and Nutritional Sciences)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March , 1994

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of Family & Nutritional Sciences

The University of British Columbia
Vancouver, Canada

Date March 29/94

DE-6 (2/88)

ABSTRACT

Human milk is relatively high in cholesterol compared with infant formula (10-15 mg/dL versus 0-1.1 mg/dL respectively). The high intake of cholesterol by breast-fed infants as compared to those fed formula has been shown in the past to result in high circulating levels of cholesterol. It is presently hypothesized that this abundance of cholesterol in the diet inhibits endogenous cholesterol synthesis, resulting in the low rates of cholesterol synthesis seen in breast-fed infants, as opposed to formula-fed infants. Continual suppression of cholesterol synthesis in infancy may render the breast-fed infant less able to synthesize cholesterol as an adult. Ideally, when challenged with a high cholesterol diet as an adult, it would be preferable for the body to limit its own synthesis of cholesterol, in order to counterbalance the increased availability of cholesterol in the diet. Infants never having had to make their own cholesterol would have an advantage over those whose metabolism had been imprinted at a young age to synthesize cholesterol. This latter group may not have the same capacity to downregulate endogenous cholesterol synthesis as adults.

Three groups of infants were used to examine the effect of cholesterol feeding in the first four months of life on serum lipids and endogenous cholesterol synthesis. Group one was breast-fed. Group two was fed a regular commercially available infant formula. Group three was fed the same formula enriched with cholesterol at a level similar to that found in breast milk (13 mg/dL). There was no difference in serum lipids between breast-fed and formula-fed infants, nor between the two different formula groups. Total-cholesterol was 4.33± 0.85, 3.65±0.40, and 3.75±1.06 mmol/L for the breast-fed, modified formula-fed, and regular formula-fed respectively. LDL-cholesterol was 2.18±0.81, 1.43±0.56, and 1.64±0.72 mmol/L respectively. HDL-cholesterol

levels were 1.17± 0.26, 1.10±0.30, 1.07±0.14 mmol/L respectively. Triglyceride levels were 4.90±1.60, 5.61±2.48, and 5.15±2.20 mmol/L respectively. Fractional synthetic rates of cholesterol (FSR) were significantly ($p<0.001$) different between breast-fed and regular formula-fed infants. Values were 2.04±1.21% per day for the breast-fed group (n=9) and 8.29±0.77% per day for the regular formula-fed group (n=3). FSR of the modified formula-fed group was 8.53±0.10% per day (n=5). There was no difference in FSR between the two formula-fed groups. Thus, addition of cholesterol to infant formula did not result in suppression of cholesterol production. Rather, the same rate of cholesterol synthesis as the regular formula-fed group was observed.

The number os subjects precluded the confidence level supported by a larger study population, hence resulting in a cautious interpretation of the data. However, from these findings it can be suggested that the form of the added cholesterol (unesterified cholesterol) was not similar enough to that found in breast milk (partially esterified cholesterol) to exert a significant effect. As well, one may further speculate that there is some other factor responsible, such as differences in fatty acid position on the triglyceride molecule between breast milk and infant formula. Even though breast milk contains significantly more cholesterol than infant formula, based on results of the present research, there is no advantage of fortifying infant formula with cholesterol.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

This was a collaborative research project with the University of Cincinnati Medical Research Center in Cincinnati, Ohio. It was supported by Wyeth-Ayerst Inc. in Philadelphia, Pennsylvania.

I would like to thank Dr. Peter Jones for the support and inspiration he provided. I would also like to thank Dr. Thor Thorkelsson, Dr. Reginald Tsang, and Susan Krug-Wispe at the Medical Research Center in Cincinnati for their support throughout the study period.

I am also grateful to Brian Toy for sharing of his computer expertise.

I INTRODUCTION

Cardiovascular heart disease has emerged as the leading cause of mortality and morbidity in North American society (Grundy, 1988). Much research has focused on the etiology and prevention of this disease, specifically in the area of cholesterol metabolism, as elevated serum cholesterol is a major risk factor for atherosclerosis. As a result, examination of factors controlling cholesterol metabolism has become important.

Cholesterol homeostasis is achieved by a balance of dietary cholesterol intake, fecal cholesterol excretion, and endogenous synthesis. Alteration of any of these factors may cause an imbalance of this system, ultimately reflecting itself in a change in body pools and serum cholesterol levels.

Various species of animals appear to vary greatly in their interplay of these mechanisms. For example, rabbits are highly sensitive and experience a dramatic increase in the amount of cholesterol in the blood in response to dietary cholesterol (Grundy *et al.*, 1969). In this case, cholesterol is absorbed much more quickly than it is removed. In rats, however, cholesterol feeding results in marked inhibition of cholesterol synthesis and enhanced excretion via bile acids.

The rate of cholesterol synthesis per kilogram of body weight varies markedly with the size of the animal (Dietschy *et al.*, 1993). For example, cholesterol synthesis by the rat is approximately 70 mg/kg/day for males and almost 120 mg/kg/day for females. The amount produced by the rabbit is approximately 40-50 mg/kg/day. The amount produced by man is only about 10 mg/kg/day, however, the response to a dietary cholesterol challenge is not so well defined as it is in animals.

Cholesterol is presented to the gut through the diet and via secretion with the bile. Safonova *et al.* (1993) defined three distinct groups of people, based on the degree to which they absorb this cholesterol: those with high, medium, and low rates of cholesterol uptake. This could

correspond to hypo and hyperresponders to cholesterol-rich diets. Further, the rate of cholesterol uptake was shown to positively correlate with the rate of intestinal cholesterol synthesis in high and medium responders. This demonstrates something other than simple negative feedback regulation of dietary cholesterol. Individuals with low rates of cholesterol uptake, however, did demonstrate very high rates of cholesterol synthesis, reflecting a compensatory mechanism of cholesterol synthesis when cell cholesterol content dropped too low (Safonova *et al.*, 1993). Miettinen and Kesaniemi (1989) also demonstrated increased absorption of dietary cholesterol with increased cholesterol intake. The greater the absorption, the less the rates of biliary secretion, fecal elimination, and cholesterol synthesis. No distinction was made between hypo and hyperresponders.

The effect of various dietary components on serum cholesterol levels has been studied extensively. The role of dietary cholesterol has been frequently evaluated in adults. In general, the effects of dietary cholesterol on cholesterol synthesis and serum lipids have not been firmly established. The infant diet may be playing a role in the variety of responses to dietary cholesterol seen in adults. Studies focusing on infants, however, are sparse. This is in part because drawing blood from infants for analysis poses difficult.

The objective of the present study was to determine if early cholesterol feeding could have an impact on endogenous cholesterol synthetic rates.

Specific objectives were as follows:

1. To compare cholesterol synthetic rate, at four months of age via deuterium incorporation methodology, in infants fed breast milk versus those fed formula containing either high or low cholesterol.

2. To compare cholesterol synthesis at four months of age, via serum lathosterol analysis, in

infants fed breast milk versus those fed formula containing either high or low cholesterol.

3. To compare circulating cholesterol levels at four months of age in infants fed breast milk versus those fed formula containing either high or low cholesterol.

4. To test the efficacy of using small blood sample sizes in analysis of cholesterol synthetic rates via deuterium incorporation methodology.

The null hypotheses for the study were as follows:

H1: There is no difference in cholesterol synthetic rate, at four months of age in infants fed breast milk versus those fed formula containing either high or low cholesterol, as measured by deuterium incorporation methodology.

H2: There is no difference in cholesterol synthesis at four months of age in infants fed breast milk versus those fed formula containing either high or low cholesterol, as measured by serum lathosterol analysis.

H3: There is no difference in circulating cholesterol levels at four months of age, in infants fed breast milk versus those fed formula containing either high or low cholesterol.

II LITERATURE REVIEW

The effect of various dietary components on serum cholesterol levels has been studied extensively. The majority of this research has focused on animals and the adult human population. Few studies have looked at the effects on human infants. Since the first signs of atherosclerosis begin in childhood (Li *et al.*, 1989), examination of factors controlling cholesterol metabolism in infancy are of interest. If cholesterol is abundant in the diet of the infant, endogenous cholesterol synthesis would not be necessary. As a result, suppression of cholesterol synthesis would be the

norm and could conceivably be imprinted in the infants' metabolism, and beneficial effects seen later in life. This is in contrast to those infants requiring high levels of cholesterol synthesis, due to low dietary cholesterol intake. In this case, a high rate of cholesterol synthesis may be an imprinted function in the infants' metabolism, potentially resulting in a decreased capacity to downregulate endogenous cholesterol synthesis in adulthood.

The effect of dietary cholesterol on specific parameters of cholesterol metabolism has produced variable results, both in human and animal studies. The need to examine the infant and the possibility of an imprinting effect of the infant diet on adult cholesterol metabolism becomes apparent. Further, the human infant presents itself as an attractive research model, due to its relatively limited diet, and consequently limited potential for other possible dietary intervening variables. Studies to date on infants have clearly shown that those who are breast-fed (cholesterol content of breast milk approximately 10-15 mg/dL), have higher serum cholesterol levels than those infants who are fed formula of lower cholesterol concentration (Friedman and Goldberg 1975; Huttunen *et al.*, 1983; Cruz *et al.*, 1993). The question remains as to whether this effect is due to the high cholesterol content of the breast milk versus formula, or to some other factor present in human milk that is not available in commercially prepared infant formulae. Further, whether these effects have any long-term implications is questionable. Previous research has failed to produce any definitive answers.

ANIMAL STUDIES:

Many animal studies have shown that the infant diet has an immediate effect on serum cholesterol levels. For example, Green *et al.* (1981) showed that rats fed a semi-synthetic diet containing 15% lard and 1.5% cholesterol, had significantly higher plasma cholesterol concentrations than rats fed either a stock diet or a semisynthetic diet containing 15% lard alone,

thus demonstrating an effect of cholesterol alone. Similar results have been shown in other studies using rats (Hahn *et al.*, 1977; Hahn and Kirby 1973; Innis 1989; Naseem *et al.*, 1980). Differences seen between groups has been suggested to be due to differences in cholesterol absorption efficiency and conversion of cholesterol to bile acids.

Controversy exists in the studies looking at the prolonged effects of early cholesterol feeding. For example, Reiser and Sidelman (1972) fed rats either a stock diet or one of several semisynthetic diets during pregnancy and lactation, to alter the cholesterol content of their milk. Pups were weaned at 30 days of age to a cholesterol-free semisynthetic diet containing 10% lard. At 60 days of age, they were fed a diet containing 60% lard and 0.5% cholesterol (i.e. a cholesterol challenge diet). Results showed that those rats whose dam's milk was lower in cholesterol had a hypercholesterolemic response to a cholesterol challenge after weaning. This seems to demonstrate an effect of early cholesterol feeding on future response to dietary cholesterol. Similar results have been demonstrated by Hahn *et al.* (1973, 1976, 1977, 1978), where rats prematurely weaned to a low cholesterol diet had higher plasma cholesterol levels than normally weaned rats. Many other studies (Kris-Etherton *et al.*, 1979; Li *et al.*, 1979, 1980; Naseem *et al.*, 1980; Reiser *et al.*, 1977; Roberts and West ,1974) demonstrated similar trends.

In the study by Green *et al.* (1981), early cholesterol feeding did not have a beneficial effect on response to dietary cholesterol in later life. That is, plasma cholesterol levels during a challenge period of high cholesterol intake were higher in rat pups previously nursed by dams fed a high fat/high cholesterol diet. Kris-Etherton *et al.* (1979) reported similar results. In their experiment, the cholesterol content of rats' milk was experimentally increased by dietary manipulation. Results showed no protective effect of increased milk cholesterol on plasma cholesterol levels in response to cholesterol feeding at five to seven months of age. Differences in percentage

cholesterol content of the dam's diets, as well as the length of time the pups were maintained on the challenge diet may have accounted for the differences seen between the studies. Differences in the polyunsaturated to saturated (P/S) ratio between various formulas used in studies may have had an effect on adult plasma cholesterol levels as well.

The piglet shares a more similar physiology with that of the human. As well, unlike other animals such as the rat, piglets exhibit altered serum cholesterol and atherosclerotic lesions on high cholesterol diets, and therefore their cholesterol metabolism is more responsive to dietary manipulation. Thus it presents itself as an attractive model to use to study this aspect further.

Jones *et al.* (1990) demonstrated higher plasma cholesterol concentrations in sow milk-fed piglets, compared with formula-fed piglets, in agreement with previous studies mentioned. In addition to this finding though, an effect on HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, was noted. Formula-fed piglets had higher HMG-CoA reductase activity in liver than sow milk-fed piglets on days 5, 10, and 15. This points to an effect of cholesterol feeding on suppressing synthetic rates of cholesterol.

It can be argued that the possibility exists that changes in milk cholesterol concentration may be accompanied by changes in caloric density of the milk. For example, increased caloric density may result in decreased intake, and therefore no changes in cholesterol intake between breast-fed and formula-fed infants, despite differences in cholesterol content between various dietary treatments. Differences observed in serum cholesterol levels or cholesterol synthetic rates between groups would therefore be due to some other factor in breast milk other than the cholesterol. In a study by Whatley *et al.* (1981), however, the milk intake of suckling rabbits was similar among all groups of pups, regardless of the cholesterol content of the mother's milk.

Further, the same effect of increased cholesterol feeding on suppression of HMG-CoA reductase activity was shown in suckling rats via maternal cholestyramine feeding (Innis, 1988).

A more recent study was carried out by Mott *et al.* (1990) in baboons. In this study, no effect of early cholesterol feeding was shown on serum cholesterol levels measured at six to eight years of life. Unlike other studies, however, significant effects were shown to occur on cholesterol metabolism. In this study ^{14}C-labelled cholesterol was administered through the diet to the baboons. Serum and fecal collections were taken to measure the radioactivity. Comparison of the radioactivity in the fecal acidic and neutral steroid fractions was measured and compared to the specific activity of plasma cholesterol, giving an indication of cholesterol turnover. Of importance here was the higher bile acid excretion rate (40% increase) in response to a high cholesterol diet, in baboons that were formula-fed as infants. Those that were breast-fed increased bile acid excretion rate only 2%. However, the response of the cholesterol production rate was especially noteworthy. Baboons breast-fed as infants showed a lower cholesterol production rate in response to a high cholesterol diet as adults. Since primates, unlike other animals, are more similar to humans in anatomy, physiology, psychology, disease susceptibility, and nutrient requirements (Grundy, 1988), results of the baboon study would appear to be more applicable to the human.

The degree to which results from animal studies may be extrapolated to humans, however, must always be questioned. "What is poison to man is not always poison to the lower animals and, on the other hand, some of the lower animals are poisoned by substances not hurtful to man" (Christison, 1832). For example, rats demonstrate differences in cholesterol metabolism from humans, such as the enhancement of bile acid formation when fed a high cholesterol diet (Li *et al.*, 1980).

A study on guinea pigs carried out by Li *et al.* (1980) showed no effect of early cholesterol feeding nor early weaning on plasma cholesterol levels in later life, when challenged with a high cholesterol diet. The use of guinea pigs as a model, however, is not ideal. Guinea pigs typically have low plasma cholesterol levels as well as low cholesterol synthetic activity in the liver. Such interspecies differences in cholesterol metabolism have resulted in variable results among different studies.

Whereas primates (i.e. monkeys, baboons, and apes) would appear to be the most appropriate animal model to use in nutrition research, the expense and lack of proper facilities limits the use of this group of animals. Due to differences between various animal species and humans, and the impracticality of importing primates for use in studies, humans are the only research model capable of providing definitive answers.

HUMAN STUDIES:

Potential effects of cholesterol feeding on various parameters of cholesterol metabolism in humans has been studied extensively. Responses to dietary cholesterol supplementation vary among adults, and consequently results have been somewhat more variable than has been demonstrated in animal studies. When modest amounts of cholesterol are added to the daily diet (eg. 2 eggs), little change in circulating cholesterol level is seen (Slater *et al.*, 1976; Kummerow *et al.*, 1977; Porter *et al.*, 1977; Flynn *et al.*, 1979; Oh and Miller 1985; Edington *et al.*, 1987; McNamara *et al.*, 1987). The major influence of such a change appears to be the baseline dietary cholesterol intake, i.e. the lower the baseline cholesterol intake, the greater the change in serum cholesterol seen with added dietary cholesterol. Addition of increasing amounts of cholesterol to the diet produces a multitude of responses among the population. That is, some individuals demonstrate no effect (Grundy *et al.*, 1969; Jones *et al.*, 1993), others a modest effect (Clifton *et*

al., 1990; McNamara *et al.*, 1987; Nestel and Poyser, 1976), and still others a more marked effect (Roberts *et al.*, 1981). For example, within a single study, addition of dietary cholesterol may result in increases in total serum cholesterol from zero to more than 100% (Quintao *et al.*, 1971; Nestel and Poyser, 1976). In a study by Li *et al.* (unpublished data), neither cholesterol intake, nor plasma cholesterol concentration appeared to have an effect on endogenous cholesterol synthesis.

Varying responses to dietary cholesterol may be due to differences in cholesterol absorption efficiency, neutral sterol excretion, conversion of hepatic cholesterol to bile acids, or effects on the activity of HMG-CoA reductase or other major enzymes involved in cholesterol biosynthesis. The degree to which any one of these factors is involved may be due to genetic or prior environmental differences, i.e. infant diet.

When challenged with a high cholesterol meal, individuals respond differently. Some increase their serum cholesterol to varying degrees while in others, levels remain relatively constant (Hopkins, 1992). Numerous studies suggest that many individuals have rather precise feedback control mechanisms regulating changes in plasma cholesterol in response to increased dietary cholesterol (Nestel and Poyser, 1976; Kummerow *et al.*, 1977; Flynn *et al.*, 1979; Mistry *et al.*, 1981; Schonfeld *et al.*, 1982; Beynen and Katan, 1985). These differences in cholesterol regulation are due in part to inter-individual differences in capacity to downregulate cholesterol synthesis. Ideally, one would want to limit endogenous synthesis of cholesterol, in order to counterbalance the increased availability of cholesterol in the diet. This ability to limit synthesis, however, appears highly variable among individuals. Some researchers believe that some people are nonresponders to diet (Jacobs *et al.*, 1983). That is, dietary treatment of high serum cholesterol results in no change in serum cholesterol levels. Nestel and Poyser (1976) found that

those who compensate for an increased dietary cholesterol intake by bile acid excretion, rather than suppression of endogenous cholesterol synthesis, demonstrate substantial increases in plasma cholesterol. This observation is in contrast to those in which the major compensatory response was via reduction in cholesterol synthesis. This latter group showed no significant rise in plasma cholesterol levels. Thus, the focus of attention turns to cholesterol synthetic rates as the potential controlling mechanism.

In a study by McNamara *et al.* (1987), increased dietary cholesterol intake resulted in compensatory decreased fractional cholesterol absorption and/or endogenous cholesterol synthesis in 69% of subjects studied. Those that responded to a high cholesterol-containing diet by increasing their plasma cholesterol levels failed to suppress endogenous cholesterol synthesis. Further, increased output of bile acids appeared to play a secondary role, and excretion of fecal neutral steroids a very minor role (Quintao and Sperotto, 1987). Other studies have shown no effect of dietary cholesterol on cholesterol synthesis (Grundy, 1969; Jones *et al.*, 1993; Jones *et al.*, in press). Variation in amount of dietary cholesterol, as well as other fats consumed may have produced the results observed. The present study focuses on the infant diet, as greater control of nutritional components of the diet are possible. This may provide one with a clearer picture of underlying processes.

MEASUREMENT OF CHOLESTEROL SYNTHESIS:

A variety of techniques have been used to measure endogenous cholesterol synthesis, all of which pose some disadvantages.

HMG-CoA Reductase Activity:

Measurement of hepatic HMG-CoA reductase activity has been used in vitro as an indicator of hepatic cholesterol synthesis (Dietschy and Spady, 1984; Carulli *et al.*, 1989). HMG-CoA

reductase, the rate-limiting enzyme in cholesterol synthesis, only gives an indication of the relative changes in rate of cholesterol synthesis in a given tissue preparation. Absolute synthetic rates are usually grossly underestimated (Carulli *et al.*, 1989). Furthermore, the method does not allow for accurate measurement of whole body cholesterol synthesis. Its use in humans is very limited, considering the degree of invasiveness required.

Sterol Balance Methods:

Whole body cholesterol synthesis has been measured via a variety of techniques. Sterol balance methods involve collection and analysis of feces for cholesterol and its metabolites (Grundy, 1969). The technique is lengthy and relies on accurate reporting of food intake and fecal collection. As well, the method operates under the assumption that a steady state of cholesterol metabolism exists. This is especially a concern if the subject is put on a cholesterol free diet, as a new steady state may take several weeks to occur (Grundy, 1969).

Measurement of Cholesterol Precursors:

Plasma levels of cholesterol precursors have been used as an indication of human cholesterol production rate. This has been based on the premise that plasma levels of different precursors to cholesterol are increased under conditions of increased synthesis of cholesterol, and conversely are decreased when synthesis is low (Bjorkhem *et al.*, 1987). Appendix one shows the scheme of cholesterol synthesis as it occurs in the body. Most cholesterol production in the liver and intestine is derived from lathosterol, and the most abundant precursor in the lipids is lathosterol (Miettinen, 1982). In human milk, however, the most abundant precursor is desmosterol. Precursors such as mevalonic acid (Parker, 1984), squalene (Nestel, 1975), or methyl sterols (Miettinen, 1982), are assumed to accumulate in the liver during cholesterol synthesis, and leak out into the plasma (Sodhi *et al.*, 1979), whereupon they can be measured. Such methods have

been shown to produce comparable results as those derived from sterol balance methods (Miettinen, 1982), however, only a relative rather than an absolute rate of cholesterol synthesis is measured. Further, they provide only an indirect indication of cholesterol synthesis. The correlation between plasma levels of precursors throughout various body tissues and HMG-CoA reductase activity has been found to be higher for free rather than esterified methyl sterols. Very high correlations have been found for lathosterol (r^2=0.83) (Bjorkhem *et al.*, 1987). In addition to this, measurement of plasma lathosterol is attractive to use, due to its relatively easy determination by simple gas liquid chromatography, without the need for time-consuming thin layer chromatography.

In humans, the lathosterol:cholesterol ratio in plasma has been found to be directly correlated with the rate of cholesterol synthesis as measured by sterol balance (Meijer *et al.*, 1992). The rationale for using plasma lathosterol as an indicator of the rate of cholesterol synthesis is based on the assumption that it leaks out of cells and is incorporated into plasma lipids at a rate proportional to that of its formation in the cholesterol synthetic pathway. It is said to be present in plasma at concentrations 1000-fold less than is cholesterol (Hamilton *et al.*, 1992). Consequently, it is viewed as a valid indicator of whole-body cholesterol synthesis. Serum lathosterol is correlated with hepatic HMG-CoA reductase activity (Bjorkhem, 1987) and with whole-body cholesterol synthesis measured by balance studies in humans (Kempen, 1988). It is also an attractive method of analysis to use because of the small amount of plasma required. Since blood samples from infants can be small, this presents itself as a useful measure in infant studies. A high ratio of plasma lathosterol:cholesterol would indicate high rates of cholesterol synthesis, whereas a low ratio would indicate the opposite.

Measurement of Radiolabelled Cholesterol Precursors:

Radiolabelled cholesterol precursors such as ^{14}C-acetate and ^{14}C-mevalonate, have provided an alternate indirect measure of cholesterol production rate (Anderson and Dietschy, 1979). As precursor substrate is necessary for cholesterol synthesis, the rate of incorporation of ^{14}C-labelled acetate or pyruvate into cholesterol can be measured. The problem of unlabelled intermediate compounds arising from unlabelled precursors and mixing with and thus decreasing the activity of the labelled intermediate compounds exists. This results in potential underestimation of cholesterol synthesis. Continuous infusion of labelled precursor becomes necessary to maintain the radioactivity of the precursor pool at a constant level, however, one cannot measure intracellular enrichment (Grundy and Ahrens Jr., 1969). This introduces potential risks associated with radioactive hazards due to the use of ^{14}C. Finally, extended periods of time are required for measurement, and only a relative rather than an absolute measurement of cholesterol synthesis is possible.

Cholesterol Kinetic Studies Using Isotope-Labelled Cholesterol:

Cholesterol kinetic studies using ^{14}C-cholesterol give more detailed information on cholesterol production rate. After administration of the isotopically labelled cholesterol, analysis of its decay indicates total body cholesterol turnover rate, as well as the rate of exchange of cholesterol between body cholesterol pools. By measuring the radioactivity of plasma cholesterol at certain time points over several weeks, the rate of decay gives an indirect measurement of cholesterol synthesis. Major disadvantages of this method are posed by the extended periods of time necessary to obtain useful data. Further, the method assumes that a steady state of cholesterol metabolism exists (Liu *et al.*, 1975), and the measurement obtained is only an indirect measurement.

The use of tritiated water avoids the problem of dilution of the cholesterol precursor pool with unlabelled substrate (Dietschy and Spady, 1984). The ^3H atoms from $^3H_20$ are incorporated into stable, non-exchangeable positions on the cholesterol molecule. Water rapidly penetrates most cell membranes in the body, and little unlabelled water is generated within cells. Consequently, the activity of the intracellular pool of tritiated water incorporated into cholesterol is assumed to equal the activity of the tritiated water found in the extracellular pool, i.e. plasma. Thus, a direct measure of cholesterol synthesis can be made. The method assumes, however, constant incorporation of label into the sterol molecule from different organs under different metabolic conditions. The major advantage of this method is the ease with which it can be measured (Dietschy and Spady, 1984). The high levels of tritium necessary in studies pose a hazard, however, resulting in unsuitability for use in humans.

Deuterium Incorporation Methodology:

The previously discussed methods have all posed certain disadvantages in measurement of cholesterol synthesis, i.e. length of time required, limitations on the use of hazardous amounts of tracer, and only indirect or qualitative measurements obtainable. The deuterium uptake method on the other hand, provides a more sensitive technique for measuring cholesterol synthesis in humans over short periods of time.

Background:

Deuterium oxide (D_20) acts as a stable isotopically-labelled precursor of cholesterol synthesis. Based on the tritiated water methodology, it measures whole body central pool cholesterol synthesis.

Deuterium was first used as a metabolic tracer in the 1930s by Schoenheimer and Rittenburg (1937), in which large doses of deuterium were used to measure fatty acid and cholesterol

synthesis. The method did not gain wide popularity, however, due to the need for time-consuming procedures, that often produced a sample in which the cholesterol was contaminated with other sterols or lipids due to lack of precise measurement techniques. The large doses of deuterium required for detection of incorporation into cholesterol further limited its use, as subjects often experience transient dizziness following its consumption.

Improved precision of measurement using isotope ratio mass spectrometry helped to refine the technique in the 1960s (Taylor *et al.*, 1966). Greater sensitivity of mass spectrometry has continued, and consequently the required level of deuterium enrichment is ten times less than amounts required in earlier studies (Schoeller *et al.*, 1983). The ability to distinguish small differences in cholesterol synthesis, along with the limited invasiveness of the procedure and absence of radioactivity, make it a useful technique for measurement of in vivo synthetic rates in humans (Jones *et al.*, 1993; Wong *et al.*, 1991).

Assumptions:

Measurement of cholesterol synthesis using this method is based on several assumptions. As with tritiated water, cell membranes are permeable to water, therefore deuterium rapidly penetrates all cell membranes in the body (Jones, 1990). Consequently, plasma water deuterium enrichment is taken as a direct indicator of the level of deuterium enrichment in the intracellular water pool, which is used for cholesterol synthesis. It is assumed that a constant fraction of the deuterium atoms incorporated into newly synthesized free cholesterol in the body will originate from plasma water. Secondly, it is assumed that cholesterol rapidly exchanges between the major sites of synthesis, i.e. the liver and small intestine, and the central pool of cholesterol. This central pool also contains cholesterol from the pancreas, spleen, kidneys, lungs and red blood cells.

During cholesterol assembly, deuterium atoms incorporate into the cholesterol molecule at a constant ratio. This newly synthesized cholesterol is thought to be part of a central pool of cholesterol that rapidly equilibrates with free cholesterol on the various plasma lipids (Goodman *et al.*, 1973). This creates an equivalent level of deuterium enrichment throughout the plasma compartment. Therefore, measurement of deuterium enrichment of plasma free cholesterol corresponds to that of the central pool.

This central pool contains cholesterol from liver and small intestinal synthesis, as well as that from red blood cells, pancreas, spleen, kidneys and lungs (Goodman *et al.*, 1973). Two additional smaller pools have much slower equilibration rates with cholesterol in the central pool (Jones, 1990). Therefore, it is reasonable to assume that the appearance of any significant amount of label from this source into the central pool during the study period will be unlikely. Hence, only deuterium entry into the central pool is measured.

During cholesterol synthesis 18 acetyl-CoA molecules, containing 36 carbon atoms in total, are used to synthesize one molecule of cholesterol, containing 27 carbon atoms and 46 hydrogen atoms (Dietschy and Spady, 1984). These hydrogen atoms are derived from three different sources. Seven come directly from water and fifteen from NADPH during reductive steps in the biosynthetic pathway. The remaining 24 are derived from cytosolic acetyl-CoA. Therefore, a total of 22 deuterium atoms could potentially be incorporated into each molecule of cholesterol, assuming the hydrogens of NADPH were labelled with deuterium, as were the hydrogens in the body water pool. Twenty-two out of a total 46 hydrogen atoms gives a fractional number of hydrogens of 0.4783. By measuring the fractional synthetic rate of cholesterol, the absolute rate of cholesterol synthesis can be calculated.

Potential Sources of Error in Using the Deuterium Incorporation Method:

Deuterium methodology relies on certain assumptions that can potentially cause either an under or overestimation of cholesterol synthesis. First, it is assumed that complete equilibration of the hydrogens of NADPH with the deuterium of deuterium labelled body water occurs. If the period of study is too short, incomplete equilibration may occur, resulting in an underestimation of cholesterol synthesis (Goodman *et al.*, 1980). Hydrogen atoms from water may also become incorporated into substrates that are ultimately used for the generation of the cytosolic acetyl-CoA pool to be used for cholesterol synthesis (Dietschy and Spady, 1984). Consequently, predicting exactly how many deuterium atoms will be incorporated into each cholesterol molecule is difficult. If too much time elapses between deuterium administration and blood specimen collection, deuterium atoms may mix with other body molecules such as acetyl-CoA, thus decreasing the ratio of deuterium to carbon atoms measured. This would result in an underestimation of cholesterol synthesis.

Second, it is assumed that all of de novo cholesterol synthesis occurs as free cholesterol in the central pool (Jones *et al.*, 1993). Further, it is assumed that exchange with the two smaller pools is minimal, due to the slower turnover rates of these pools. If significant exchange were to occur between the different pools, dilution of the central pool with unlabelled cholesterol would occur. The result would be an underestimation of cholesterol synthesis. As previously mentioned, the time factor between deuterium administration and specimen collection is important.

Studies on Cholesterol Synthesis using Deuterium:

Studies of cholesterol synthetic rates in infants is lacking, possibly due to the nature of the analyses required. Deuterium incorporation methodology, however, has emerged as a sensitive measure of cholesterol synthesis, useable in humans over short periods of time.

Cruz *et al.* (1992) studied cholesterol synthetic rates in infants at four months of age. The infants had either been exclusively breast-fed (cholesterol content approximately 10-15 mg/dL), or formula-fed with either a soy-based product containing no cholesterol, or a cow milk-based formula containing 1.1 mg cholesterol/dL. A fourth group was added in which the diet consisted of a modified soy-based formula with added cholesterol to equal that present in the cow milk-based formula. The breast-fed infants, having the highest intake of cholesterol, demonstrated the lowest cholesterol synthetic rates (2.62±0.38% per day), as measured by deuterium incorporation methodology. This group was significantly different from the formula-fed groups. The soy-based formula-fed group had the highest synthetic rates of cholesterol (9.40±0.51% per day). Those infants consuming cows milk-based formula had a lower cholesterol synthetic rate (6.90±0.48% per day), and those consuming the modified soy-based formula had cholesterol synthetic rates intermediate between the two (8.03±0.28% per day). There was a significant ($p=0.03$) difference between infants fed soy formula versus those fed modified soy formula. These results demonstrate an effect of cholesterol on endogenous cholesterol synthesis. The question remains as to whether it is the cholesterol content of the breast milk alone which is having the effect, or some other factor, or a combination of factors. To test this hypothesis further, it would be necessary to test a group of infants consuming formula with added cholesterol in amounts more physiologic to that of breast milk.

III EXPERIMENTAL DESIGN AND METHODS

This was a collaborative study with the University of Cincinnati Medical Research Center in Cincinnati, Ohio. Seventeen term healthy infants (2642-3700 gm, gestational age 37-41 weeks as determined from last normal menstrual cycle), were recruited into three treatment groups, differing in type of diet: one breast-feeding group (n=9), one regular cow milk-based formula group (n=3), and one modified cow milk-based formula group with added cholesterol (n=5). Both males and females were studied. Whereas both black and white infants were included, only infants whose parents were of the same race were accepted. There were four exclusion criteria:

1. Evidence of cardiac, respiratory, hematologic, gastrointestinal, or other systemic disease in the infants.
2. History of hypercholesterolemia and/or hypertriglyceridemia in the mother, father, and/or sibling.
3. Infants initially recruited who subsequently developed formula intolerance requiring change of formula (brand or type), as advised by the physician in charge of their care.
4. Infants of breast-feeding mothers who were strict vegetarians or vegans.

Recruitment took place in the order in which the infants were entered in the admission log book at the hospitals. The formula-fed infants were randomized to either of the two different formulae, using a computer-generated random numbers table. This allowed double-blinding of subject groups. Drop-out subjects, of which there was one, were replaced by the next eligible infant of the same sex and race.

Due to the nature of the treatment, complete randomization was not practical. That is, the decision on whether to breast-feed or formula-feed rested with the mother. For the breast-feeding

group, only those mothers who planned to breast-feed exclusively for at least the first four months of life were invited to participate in the study. For the formula-fed groups, only infants of those mothers who had made a prior decision to use cow milk-based formula (as opposed to soy milk-based formula or breast milk), as documented on the admission notes prior to delivery, were recruited. Formulas were provided by Wyeth-Ayerst in Philadelphia, Pennsylvania.

The infants in each group consumed a milk diet exclusively for the four month test period. At the end of four months, a three day blood draw took place to be used for measurement of cholesterol synthetic rate, as well as serum lipids. A four month period was chosen to avoid potential confounding effects of other foods or supplementary milk that is generally introduced into the diet of infants after this age (Ernst *et al.*, 1990), thus allowing evaluation of the effect of the cholesterol content of the milk diet on measured parameters of cholesterol metabolism.

Demographics were collected on the infants at entry into the study and at the four month test period, i.e. weight. At four months, a three day blood draw took place. On day one, the infants were weighed and a blood sample obtained for determination of baseline deuterium enrichment. Five-hundred mg/kg of deuterium oxide was then administered by mouth to the infant, using a disposable syringe. Further blood samples were obtained on days two and three for determination of postloading deuterium excess enrichment. A maintenance oral dose of 50 mg/kg of deuterium oxide followed collection of the blood sample on day two. No more than 8 mL of blood was drawn for each test day. All samples were drawn between 9 AM and 12 noon. Infants maintained normal feeding patterns throughout the study. Fasting before blood draws was not required, as mothers may have been uncomfortable with withholding milk from the infants.

Each blood sample was fractionated. The serum was used for lathosterol analysis, as well as for determination of lipid profiles (serum cholesterol, triglycerides, HDL-cholesterol, and LDL-

cholesterol), by the group in Cincinnati. Methods used were validated by NIH-Lipid Research Clinics. The red blood cell fraction was sent to the University of British Columbia for determination of cholesterol synthesis. Two methods were used for this determination: Deuterium uptake methodology as developed by Jones *et al.* (1993), and plasma lathosterol determination based on the methods of Bjorkhem (Bjorkhem *et al.*, 1987).

Laboratory Analysis:

Determination of Cholesterol Synthesis by Deuterium Uptake Methodology

1. Lipid Extraction From Red Blood Cells:

Duplicates of 1.0 and 1.5 mL red blood cell samples were used. Six mL of methanol was added and samples were heated in a 55°C water bath for 15 minutes. Hexane/chloroform (4:1 v/v) was added and the mixture shaken for 15 minutes, upon which 0.75 mL distilled water was added and shaken again for another 10-15 minutes. This was followed by centrifugation for 15 minutes at 1500 g. The upper hexane-chloroform layer containing the lipid was removed. Lipid was re-extracted, using hexane-chloroform, upon which the upper layer was again removed, and combined with the first. The solvent was then evaporated by drying under nitrogen, leaving the lipid extract.

2. Purification of Cholesterol by Thin Layer Chromatography (TLC):

The extract was redissolved in a small amount of chloroform and streaked onto thin-layer silica gel plates, which were prepared in the lab. Plates were developed in petroleum ether/ethyl ether/acetic acid (135:15:1.5 v/v/v) for approximately one hour and dried in air. Lipid bands were visualized in iodine vapour against a cholesterol standard. The free cholesterol band, which was the one of interest, presented itself as the first band from the bottom. This band was scraped from the silica plate and extracted three times using 6 mL, 4 mL, and 3 mL respectively of

hexane/chloroform/ether (5:2:1 v/v/v). The pooled solvent was dried under nitrogen, leaving the purified lipid extract.

3. Combustion: Conversion of Cholesterol to Water and Carbon Dioxide:

The extract containing the free cholesterol was redissolved in a small amount of chloroform and transferred into a Pyrex combustion tube, 18 cm in length (Corning Glass Works, Corning, New York). Ground copper oxide (0.5 gm) (BDH, Toronto, Ontario) and a 2-2.5 cm piece of silver wire (1 mm diameter) were added to each tube. The solvent was then removed by gradual heating under vacuum until it had boiled off, leaving the cholesterol. Pressure was allowed to return to baseline, upon which tubes were sealed under vacuum using a hydrogen torch. Tubes then underwent combustion at 540°C for four hours, to produce water and carbon dioxide, upon which they were allowed to slowly cool to room temperature.

4. Distillation: Reduction of H_20/D_20 to Hydrogen/Deuterium Gas:

In brief, the carbon dioxide produced during combustion was removed under vacuum, and the water produced distilled into another Pyrex tube containing 60 mg zinc shavings (Biogeochemical Labs, Indiana University, Bloomington, IN). After allowing pressure to return to baseline, tubes were sealed under vacuum using a hydrogen torch. Tubes then underwent reduction at 540°C for exactly 30 minutes, to produce hydrogen-deuterium gas.

5. Preparation of Plasma Samples:

Samples of plasma (100 uL), were diluted with Vancouver tap water two-fold for baseline and six-fold for the following two blood draws. When plasma was not available, red blood cells were used. A 100 uL sample was diluted with Vancouver tap water 1.4-fold for baseline, and 2.8-fold for the following two blood draws. From these, 2 uL samples were distilled into Pyrex tubes, containing 60 mg zinc. Tubes were sealed under vacuum and reduced in a 540°C oven for exactly

30 minutes to produce hydrogen-deuterium gas from the plasma or red blood cell water. Dilutions were necessary in order to produce enrichments within the range of standards used with the mass spectrometer.

6. Gas-Isotope-Ratio Mass Spectrometry:

The deuterium to hydrogen ratio of the cholesterol sample was determined on a differential isotope ratio mass spectrometer (Nuclide 3-60 H/D;MAAS, Bellefonte, Pa). Reduction gas was loaded directly into the instrument. Samples were compared with two gas standards with electrical H_3^+ compensation, in order to determine ratios. Values were expressed relative to the enrichment of standard mean ocean water (SMOW) in parts per mil.

Calculations

Deuterium enrichment of red blood cell free cholesterol as described earlier, is an indicator of endogenous cholesterol synthesis. The fractional synthetic rate (FSR) was calculated in percentage per day as follows:

$$FSR\ (\%\ per\ day) = \frac{24\ hours/day \times 100 \times slope\ (at\ \%\ excess/hour)}{0.4783 \times body\ water\ ^{2}H\ (at\ \%\ excess)}$$

The slope is the difference in cholesterol enrichment above baseline versus time. The constant 0.4783 is the ratio of hydrogen atoms in the synthesized cholesterol molecule derived from body water (22), to the total number of hydrogen atoms in the cholesterol molecule (46).

Body water ^{2}H is the mean plasma enrichment over the entire three day period, corrected for baseline deuterium enrichment.

Plasma Lathosterol Determination

1. Saponification:

Plasma samples (200 uL) were combined with 50 uL of 5-alpha cholestane (0.5 ug in chloroform) and 3 mL of methanolic KOH (94 mL methanol + 6 mL 50% KOH w/v). The samples were vortexed and put on an 80°C heating block for 16-17 hours. Cooling was allowed, and samples then adjusted to 50% methanol by the addition of 2.65 mL distilled water. Petroleum ether (3 mL) was added and the mixture shaken for 10 minutes, then centrifuged for 10 minutes at 1500 rpm, upon which the upper layer was removed. Two more series of extractions followed, as in the above fashion, and the extracts pooled. Samples were dried down under nitrogen and transferred to microvials by dissolving in a small amount of petroleum ether. Samples were dried down again and then sealed. This was the semi-purified sterol fraction.

2. Silyation:

This process increased the volatility of the lathosterol. Samples were silyated with hexamethyldisilane:trimethylchlorosilane:dimethylformamide (20:2:5 v/v/v) and allowed to sit for 5 minutes at room temperature. The sterols were then analyzed by GLC. Samples were injected at 80°C and held for one minute. The oven temperature was programmed to increase to 120°C (20°C/minute), hold for 15 minutes, rise to 269°C (20°C/minute) and hold for 25 minutes, to elute the majority of the plant sterols. The oven was heated to 320°C 20°C/minute) for 5 minutes to burn any contaminants off the column.

The injector temperature was set at 300°C, and the detector temperature at 320°C. The flow rate of the carrier gas, helium, was 1 mL./minute. The split vent flow rate was 4.5 mL/minute and the purge vent 3-5 mL/minute. The column used was Restek RTx-1, 30 m, 0.25 mm ID, 0.25 u, catalogue #10123, lot #3894. The lathosterol peak identification was determined using a

standard. Calculations were done on the basis of ug lathosterol/200 uL plasma and plasma lathosterol reported as the ratio of lathosterol:cholesterol.

Statistical Analysis

Standard t-tests were run to compare the breast fed group to the formula fed group and the two formula-fed groups to each other for FSR, lathosterol:cholesterol ratio, plasma lathosterol. T-tests were also used to compare breast-fed infants to formula-fed infants for serum total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides.

Regression analysis was used to measure correlations between FSR, plasma lathosterol, lathosterol:cholesterol ratios and serum lipids - i.e. total serum cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. Regression analysis was also used to compare body weight to FSR, plasma lathosterol, and lathosterol:cholesterol ratios. Regression analysis was further used to compare the effect of breast feeding and formula feeding on weight gain and percentage weight gain.

The level of significance was set at $p<0.05$ for all tests. All statistical analysis procedures were done using SYSTAT version 5.02.

IV RESULTS

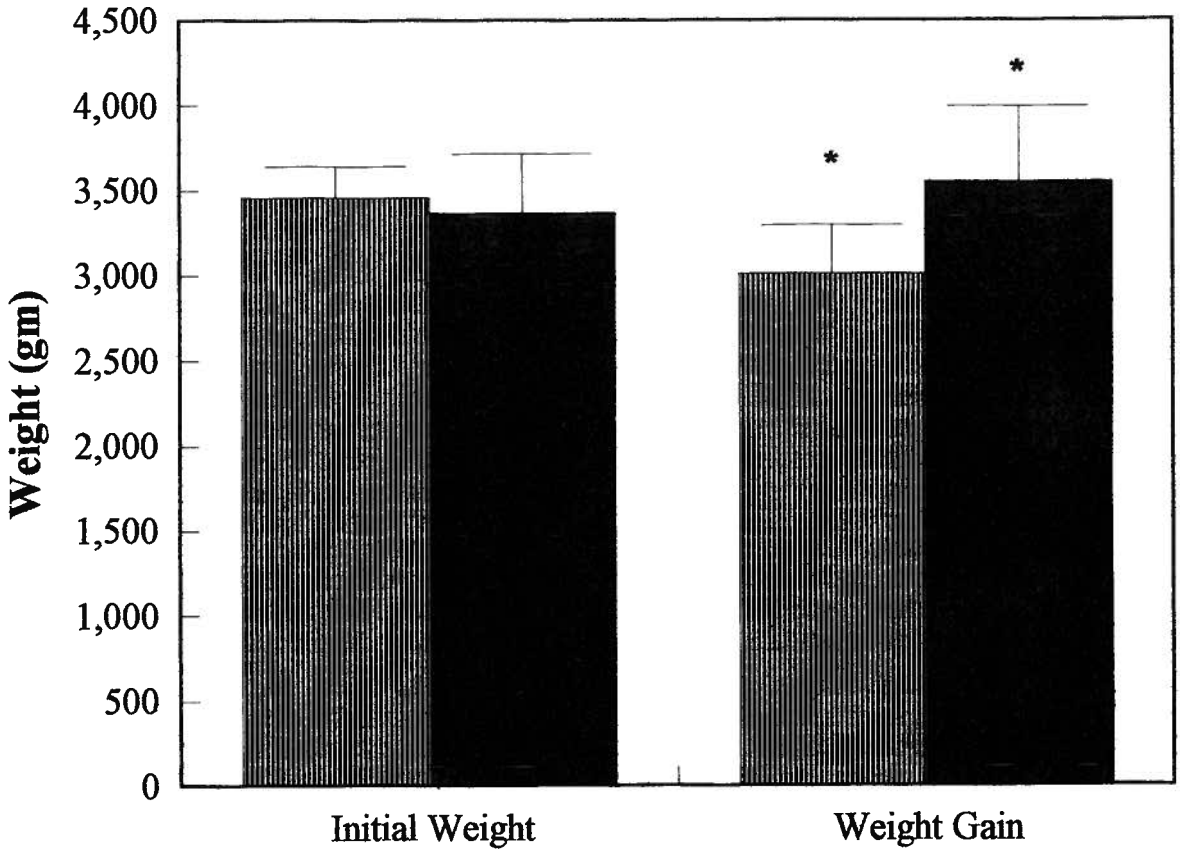
1. SUBJECT CHARACTERISTICS

At the time of analysis, 17 infants had reached four months of age - 9 breast-fed and 8 formula-fed. The breast-fed group included three white males, five white females, and one black female. The formula-fed group was made up of three infants on the regular cow milk-based formula, and five infants on the modified cow milk-based formula. The regular formula group included one white male, one black male, and one black female. The modified formula group included five white males. All infants had a gestational age of 37-40 weeks (average age = 39 weeks). Average birthweight was 3418 grams (range = 2642 gm to 3700 gm) - see Table 1. Group averages were 3456±184 grams for the breast-fed group and 3368±348 grams for the formula-fed group. Average total weight gain (measurements taken at approximately four months), was 3011±283 grams for the breast-fed group and 3552±440 grams for the formula-fed group.

2. RELATIONSHIP OF DIET TO WEIGHT GAIN

Figure1 demonstrates birthweight and weight gain of the breast-fed and formula-fed infants. There was a significant ($p=0.037$) relationship between diet and weight gain. Infants' weights were taken at four months of age ± 3 weeks. When differences in age at measurement were taken into account, formula-fed infants gained more weight than breast-fed infants. However, when taken as a percentage weight gain of birthweight, there was no significant ($p=0.163$) difference.

FIGURE 1 - Birthweight and weight gain (at 4 months) of breast-fed and formula-fed infants



mean ± standard deviation
* p = 0.037

TABLE 1 - Birthweight and weight gain of breast-fed and formula-fed infants

DIET	SEX	BIRTHWEIGHT (gm)	WEIGHT GAIN (gm)	AGE
Breast-fed:	male	3250	3380	4 months
	male	3180	2925	4 months
	male	3563	2942	4 months + 1 week
	female	3700	2680	4 months
	female	3345	3450	4 months
	female	3565	3260	4 months
	female	3400	2780	4 months
	female	3410	2935	4 months
	female	3690	2750	4 months
Mean:		**3456 ± 184**	**3011 ± 283**[a]	
Formula-fed:*	male	3610	3775	3 months + 3 weeks
	male	3570	3125	4 months
	male	3435	3875	4 months + 3 weeks
	male	2642	3158	4 months + 2 weeks
	male	3255	3080	4 months + 2 weeks
	male	3650	3635	4 months
	female	3415	4215	4 months
Mean:		**3368 ± 348**	**3552 ± 440**[a]	

Mean ± standard deviation

* Data available for only 7 subjects

[a] $p = 0.037$

3. EFFECT OF DIET ON PLASMA LIPIDS

Table 2 and Figure 2 summarize the different plasma lipids of the breast-fed and formula-fed infants. Total cholesterol was 4.33±0.85, 3.65±0.40, and 3.75±1.06 mmoL/L for the breast-fed, modified formula-fed, and regular formula-fed groups respectively. There was no statistically significant (p=0.106) difference between the breast-fed and two formula-fed groups as a whole, nor between the two formula-fed groups (p=0.857).

Plasma LDL-cholesterol was 2.18±0.81, 1.43±0.56, and 1.64±0.72 mmoL/L for the breast-fed, modified formula-fed, and regular formula-fed groups respectively. There was no statistically significant (p=0.079) difference between the breast-fed and two formula-fed groups as a whole, nor between the two formula-fed groups (p=0.663).

Plasma HDL-cholesterol was 1.17±0.26, 1.10±0.30, and 1.07 ±0.14 mmoL/L for the breast-fed, modified formula-fed, and regular formula-fed groups respectively. There was no statistically significant (p=0.472) difference between the breast-fed and two formula-fed groups as a whole, nor between the two formula-fed groups (p=0.885).

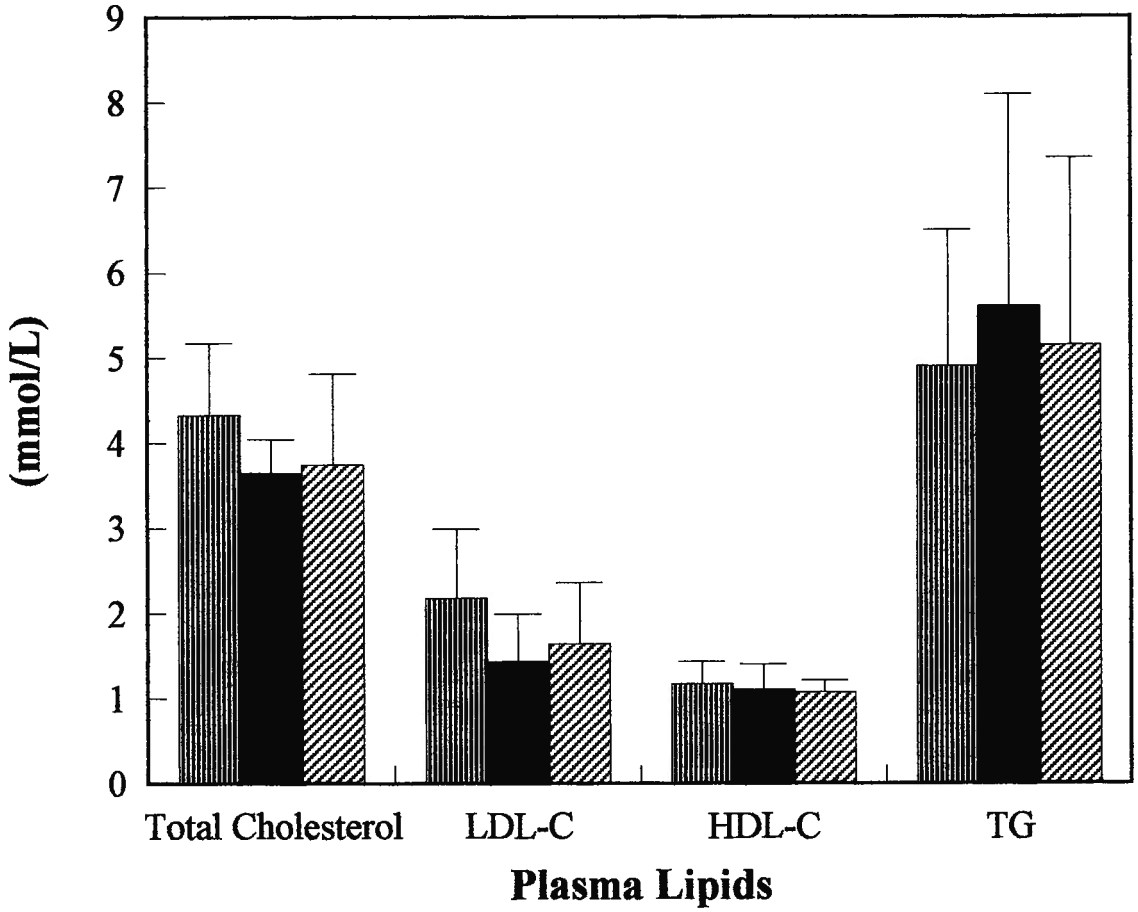
Plasma triglycerides were 4.90±1.60, 5.61±2.48, and 5.15±2.20 mmoL/L for the breast-fed, modified formula-fed, and regular formula-fed groups respectively. There was no statistically significant (p=0.568) difference between the breast-fed and two formula-fed groups as a whole, nor between the two formula-fed groups (p=0.805).

TABLE 2 - Effect of diet on plasma lipids (mmol/L) in 4 month old infants

DIET	SEX	TOTAL -C	LDL -C	HDL-C	TG
Breast-fed:	male	4.48	2.59	1.22	3.39
	male	4.51	2.07	1.04	6.97
	male	3.71	1.84	0.80	5.29
	female	3.96	1.55	1.48	4.72
	female	4.09	1.71	1.24	5.65
	female	3.76	1.97	1.24	2.69
	female	4.17	1.76	1.00	7.12
	female	6.45	4.19	1.63	3.11
	female	3.78	1.81	0.96	5.10
Mean:		**4.33 ± 0.85**	**2.18 ± 0.81**	**1.17 ± 0.26**	**4.90 ± 1.60**
CV		0.197	0.376	0.222	0.326
Formula-fed Modified:	male	3.58	1.71	1.14	3.63
	male	3.96	1.92	1.24	4.07
	male	3.01	0.49	0.62	9.48
	male	3.73	1.50	1.40	4.15
	male	3.99	1.55	1.09	6.71
Mean:		**3.65 ± 0.40**	**1.43 ± 0.56**	**1.10 ± 0.30**	**5.61 ± 2.48**
CV		0.109	0.385	0.266	0.422
Formula-fed Regular:	male	4.87	2.28	1.09	7.46
	female	2.77	0.86	0.93	4.92
	female	3.60	1.79	1.19	3.08
Mean:		**3.75 ± 1.06**	**1.64 ± 0.72**	**1.07 ± 0.14**	**5.15 ± 2.20**
CV		0.282	0.439	0.123	0.427

Mean ± standard deviation
Total-C = total cholesterol
LDL-C = low density lipoprotein cholesterol
HDL-C = high density lipoprotein cholesterol
TG = triglyercides
CV = coefficient of variation

FIGURE 2 - Effect of diet on plasma lipids in 4 month old infants



mean ± standard deviation

Breast -fed (n=9)
Formula-fed Modified (n=5)
Formula-fed Regular (n=3)

4. EFFECT OF DIET ON CHOLESTEROL SYNTHESIS

Appendix 6 shows deuterium uptake to be linear over the 0-24 hour period. After this time, the body began to lose excess deuterium, thus the uptake rate begins to exhibit curvature. Consequently 24 hour FSR values were utilized for analysis.

Table 3 and Figure 3 summarize the cholesterol synthetic rate values, as measured by deuterium uptake methodology. Breast-fed infants had a significantly ($p<.001$) lower rate of cholesterol synthesis (2.04±1.21% per day) than the two formula-fed groups as a whole (8.53±1.10 and 8.29±0.77 % per day for the modified and regular formula-fed groups respectively). There was no significant ($p=0.757$) difference between the two formula-fed groups.

Table 4 and Figure 4 summarize the plasma lathosterol and plasma lathosterol:cholesterol ratios. Mean values were 256±107 ug/dL and 0.295 x 10^{-2}±0.122, 230±104 ug/dL and 0.275 x 10^{-2}±0.089, 317±104 ug/dL and 0.414 x 10^{-2}±0.184 for the breast-fed, modified formula-fed, and regular formula-fed respectively. There was no significant ($p=0.926$ and 0.613 respectively) difference between the breast-fed and two formula-fed groups as a whole, nor between the two formula-fed groups ($p=0.197$ and 0.462 respectively).

There was no relationship found between FSR and plasma lathosterol levels ($r2=0.023$, $p=0.577$) - see Figure 5.

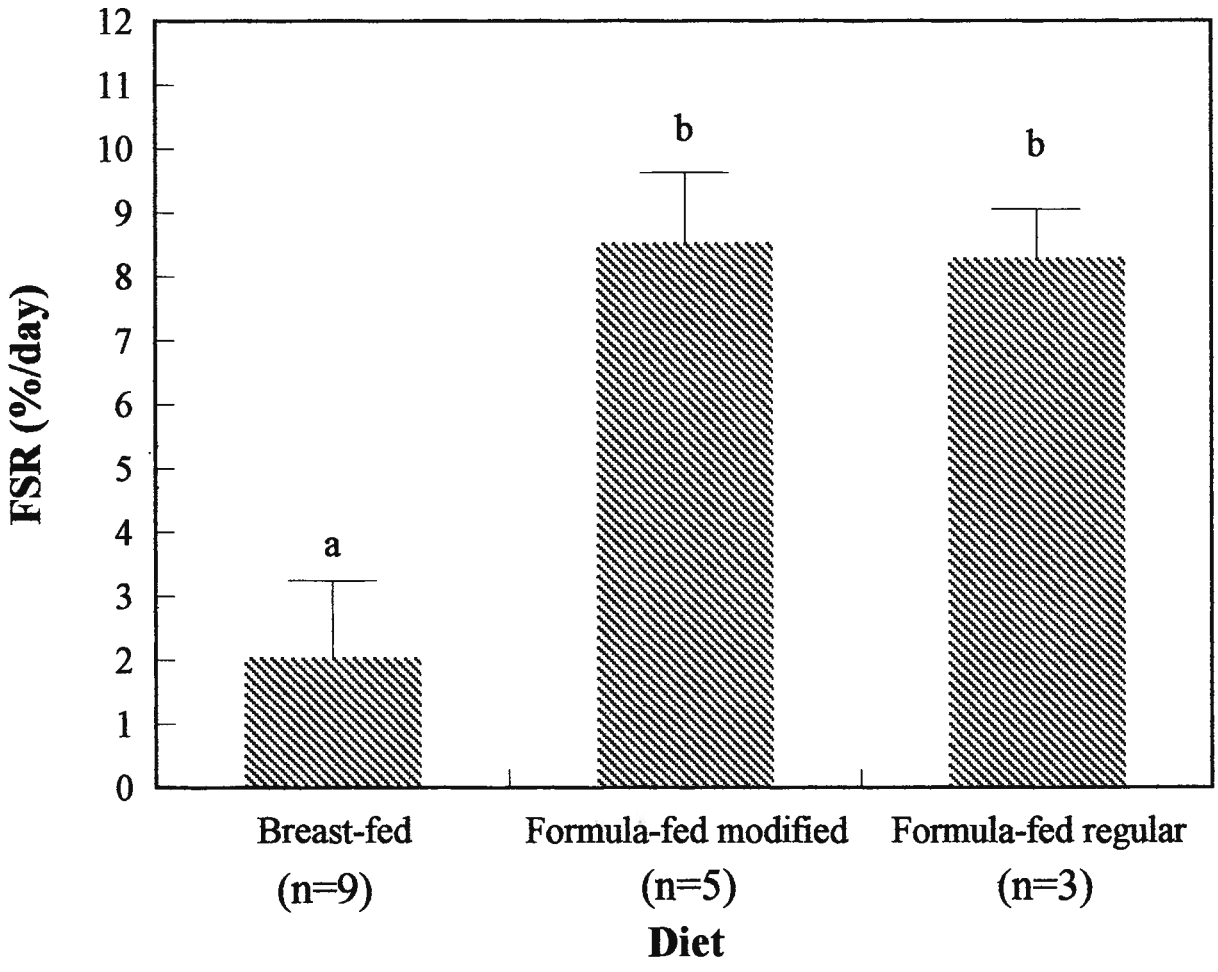
There was no relationship found between plasma lathosterol levels and plasma total cholesterol ($r2=0.124$, $p=0.182$) - see Figure 6.

TABLE 3 - Effect of diet on cholesterol synthesis (FSR) of 4 month old infants as measured by deuterium incorporation methodology

DIET	% D2O UPTAKE 0-24 hours	0-48 hours	0-72 hours	PLASMA WATER 0-24 hours	0-48 hours	0-72 hours	FSR %/day
Breast-fed:	-132.894	-67.557	-56.204	-9.093	430.556	420.207	3.96
	-87.694	-53.378	-31.065	46.944	422.075	390.751	2.61
	-163.867	-124.511	-110.378	37.742	384.556	384.213	3.17
	-53.837	-60.909	-52.858	-22.102	435.763	423.107	0.00
	-132.163	-118.501	——	79.279*	303.535*	——	2.16
	-48.096	27.671		-8.978	396.742	——	0.67
	-76.577	-54.207	-38.839	-18.047	410.536	357.826	1.89
	-112.006	-89.126	-105.815	-34.854	440.896	417.670	1.52
	-79.779	-50.465	160.809**	-15.814	353.561	258.162	2.37
Mean:							**2.04 ± 1.21**
Formula-fed Modified:	-136.186	-18.379	22.801	-22.175	334.516	306.570	9.10
	-170.426	-38.560	46.390	-2.292	570.746	520.083	7.17
	-202.532	-82.580	46.250	34.019	399.548	354.691	9.48
	-193.262	-97.012	41.029	24.634	396.480	361.648	7.52
	-191.056	-18.999	44.845	53.698	541.482	535.025	9.38
Mean:							**8.53 ± 1.10**
Formula-fed Regular:	-103.086	-83.994	——	25.114	390.150	——	7.41
	-163.653	-54.146	-21.124	-28.849	323.936	290.364	8.82
	-188.628	-83.833	-26.640	15.248	374.048	338.238	8.65
Mean:							**8.29 ± 0.77**

Mean ± standard deviation
* Red blood cell water
** 0-96 hours
D2O = deuterium
FSR = fractional synthetic rate of cholesterol
FSR of breast-fed vs formula-fed $p < 0.01$

FIGURE 3 - FSR of breast-fed and formula-fed infants at 4 months



mean ± standard deviation
a vs b - $p < 0.01$

TABLE 4 - Effect of diet on plasma lathosterol and plasma lathosterol:cholesterol ratios of 4 month old infants

DIET	PLASMA L:C	LATHOSTEROL		
	(x 10^{-2})	µg/200µL	µg/dL	µmol/L
Breast-fed:	0.342	0.004	200	5.18
	0.443	0.005	250	6.48
	0.241	0.007	350	9.07
	0.140	0.004	200	5.18
	0.389	0.004	200	5.18
	0.443	0.010	500	12.95
	0.264	0.004	200	5.18
	0.296	0.005	250	6.48
	0.101	0.003	150	3.89
Mean:	**0.295 ± 0.122**	**0.005 ± 0.002**	**256 ± 107**	**6.62 ± 2.78**
CV	0.415	0.420	0.420	0.420
Formula-fed modified:	0.218	0.004	200	5.18
	0.203	0.003	150	3.89
	0.389	0.008	400	10.36
	0.365	0.005	250	6.48
	0.202	0.003	150	3.89
Mean:	**0.275 ± 0.089**	**0.005 ± 0.002**	**230 ± 104**	**5.86 ± 2.68**
CV	0.339	0.451	0.451	0.451
Formula-fed regular:	0.536	0.007	350	9.07
	0.202	0.004	200	5.18
	0.505	0.008	400	10.36
Mean:	**0.414 ± 0.184**	**0.006 ± 0.002**	**317 ± 104**	**8.20 ± 2.70**
CV	0.445	0.329	0.329	0.329

Mean ± standard deviation
L:C = lathosterol:cholesterol ratio
CV = coefficient of variation

FIGURE 4 - Plasma lathosterol and plasma lathosterol:cholesterol of breast-fed and formula-fed infants at 4 months

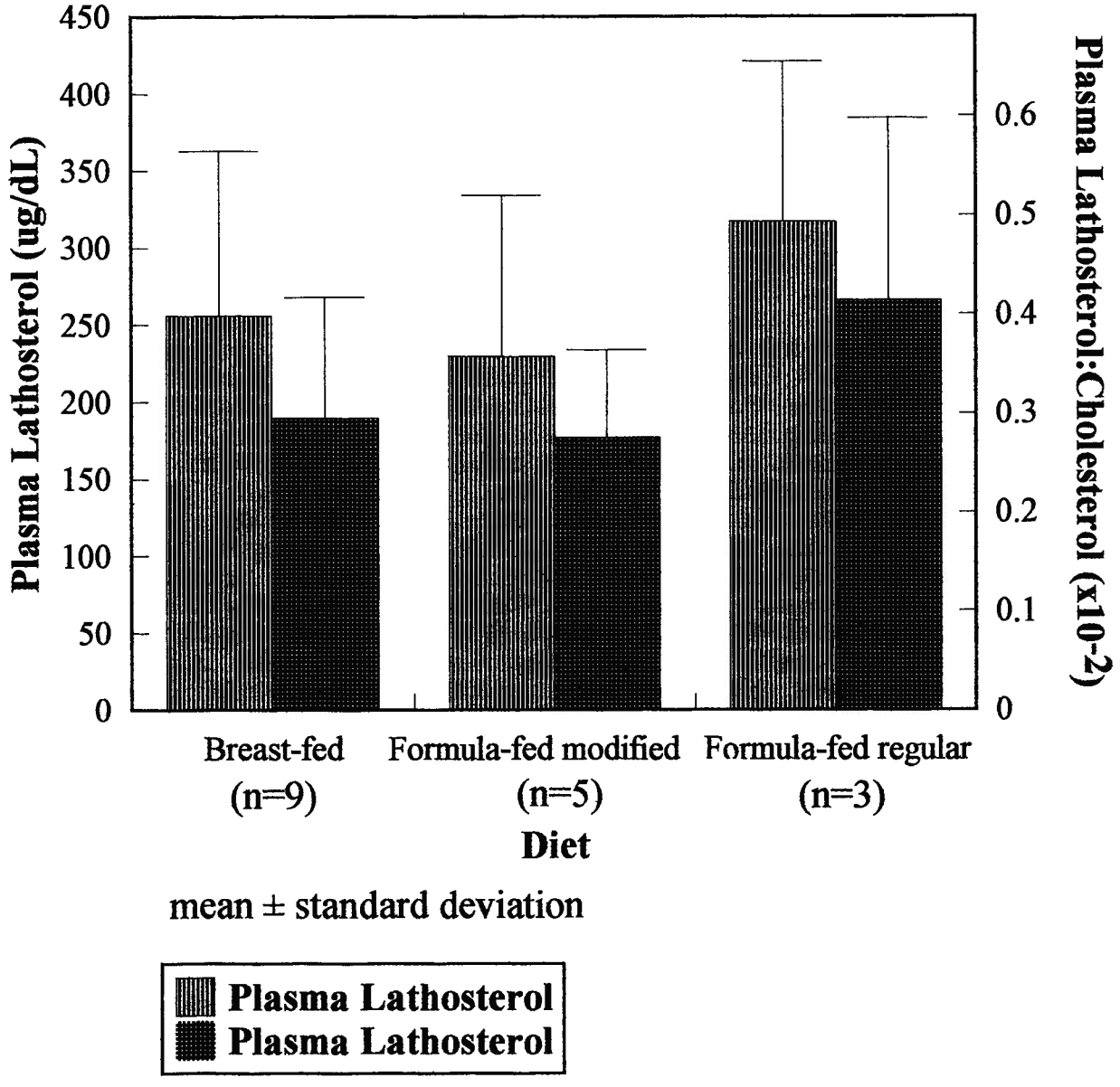


Figure 5 - FSR vs plasma lathosterol of breast-fed and formula-fed infants at 4 months

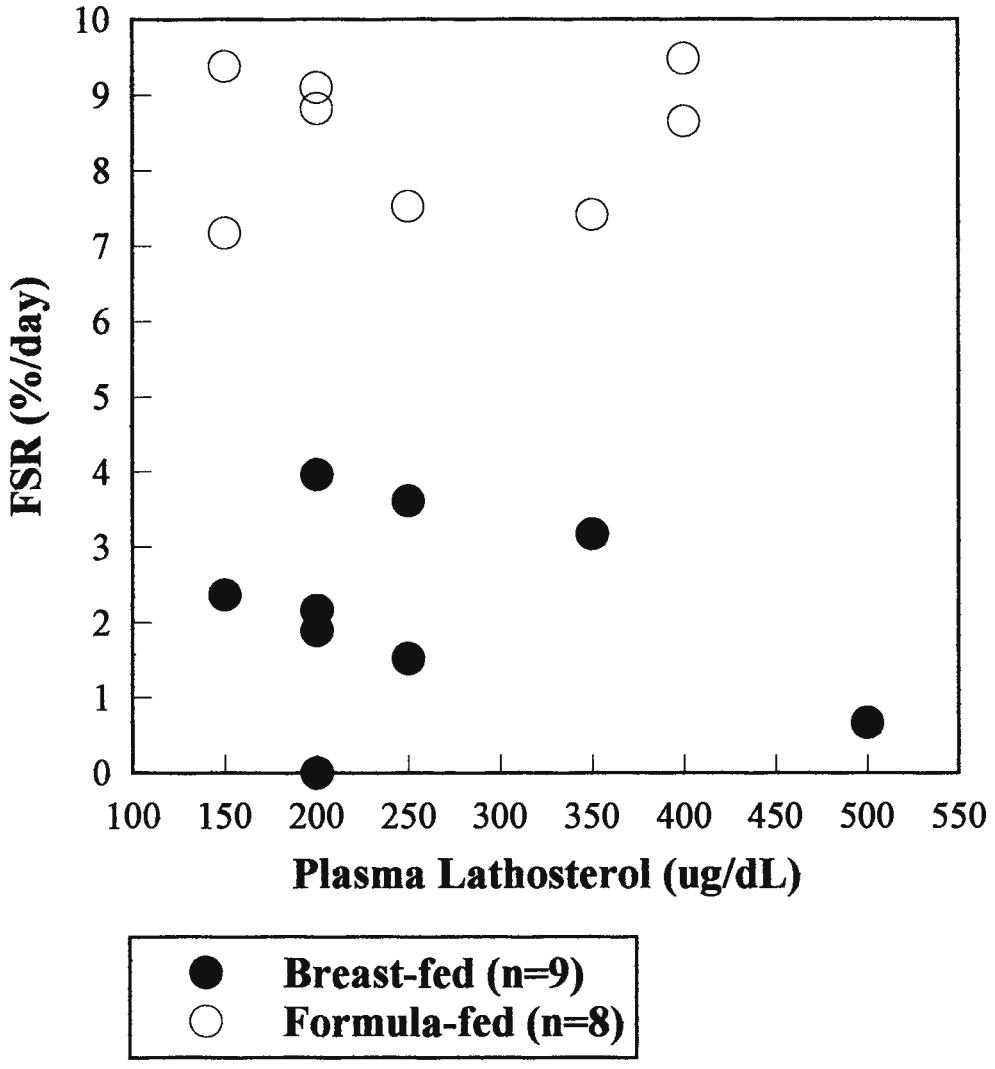
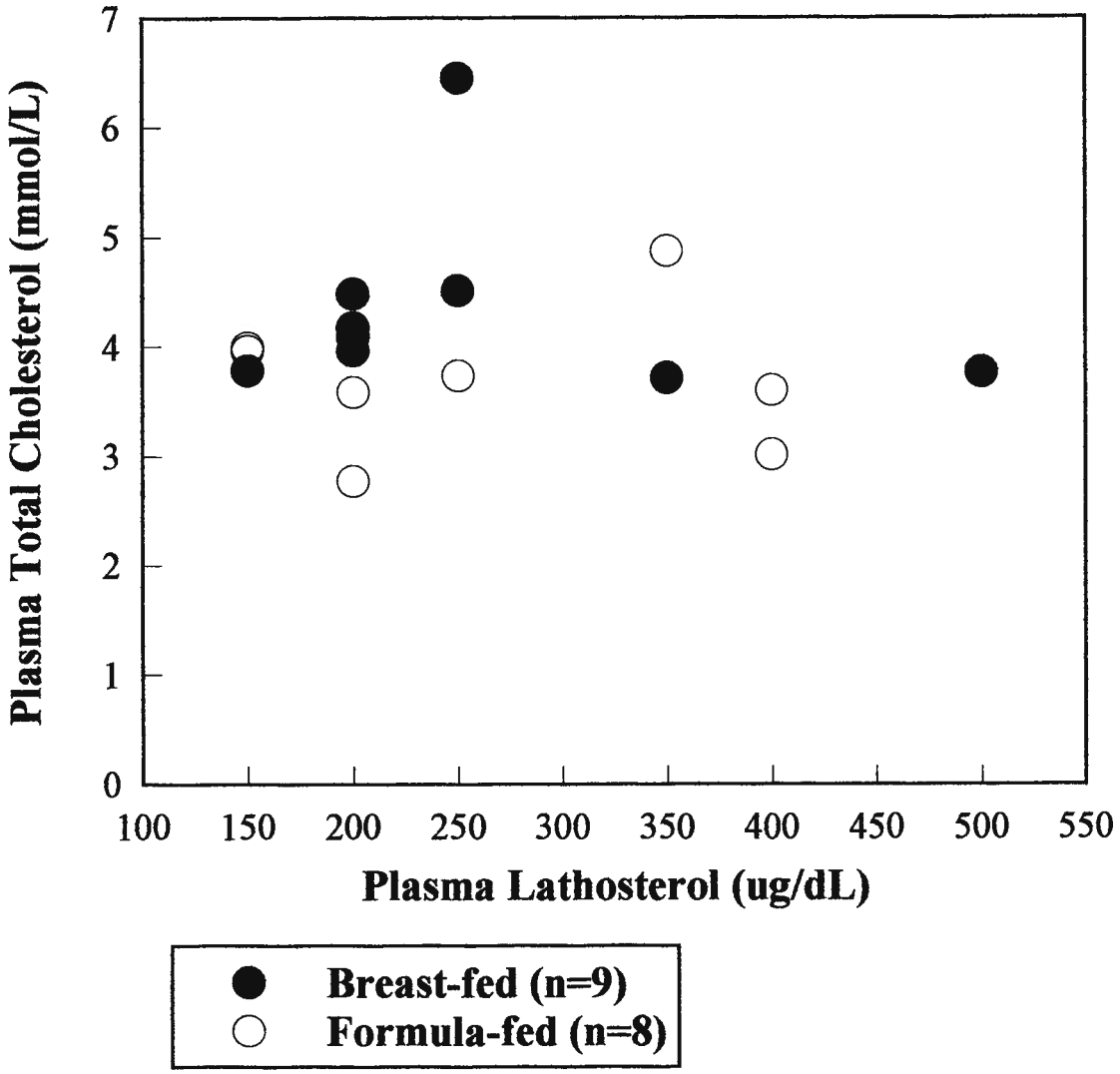


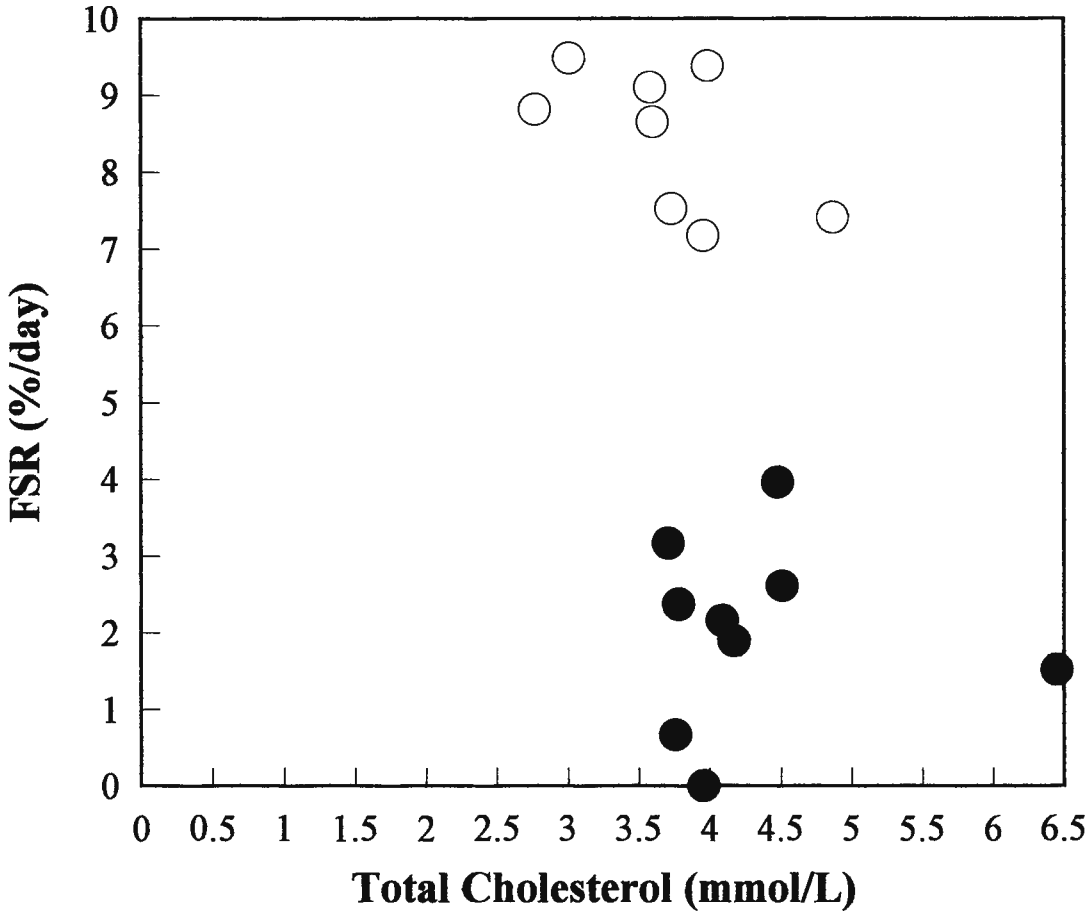
FIGURE 6 - Plasma total cholesterol vs plasma lathosterol of breast-fed and formula fed infants at 4 months



5. RELATIONSHIP OF CHOLESTEROL SYNTHESIS TO SERUM LIPIDS

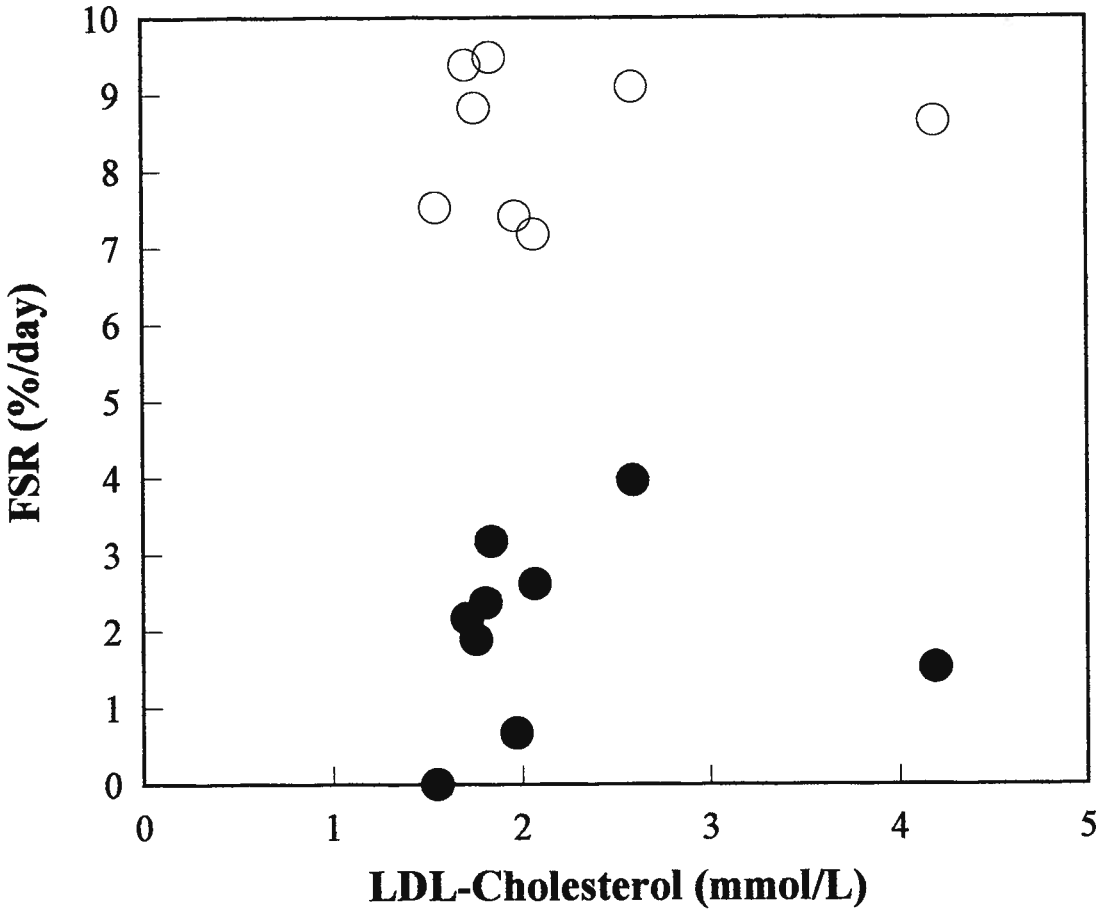
Looking at the breast-fed and two formula-fed groups as a whole, there was no significant ($p=0.10$ and 0.069 respectively) relationship between FSR and each of plasma total cholesterol and LDL-cholesterol - see Figures 7 and 8. As well, the same type of analysis showed no significant ($p=0.182$ and 0.324 respectively) relationship between plasma lathosterol and each of plasma total-cholesterol and LDL-cholesterol - see Figures 9 and 10.

FIGURE 7 - FSR vs total cholesterol of breast-fed and formula-fed infants at 4 months



FSR = fractional synthetic rate of cholesterol

FIGURE 8 - FSR vs LDL-cholesterol of breast-fed and formula-fed infants at 4 months



FSR = fractional synthetic rate of cholesterol

● **Breast-fed (n=9)**
○ **Formula-fed (n=8)**

Figure 9 - Plasma lathosterol vs plasma total cholesterol

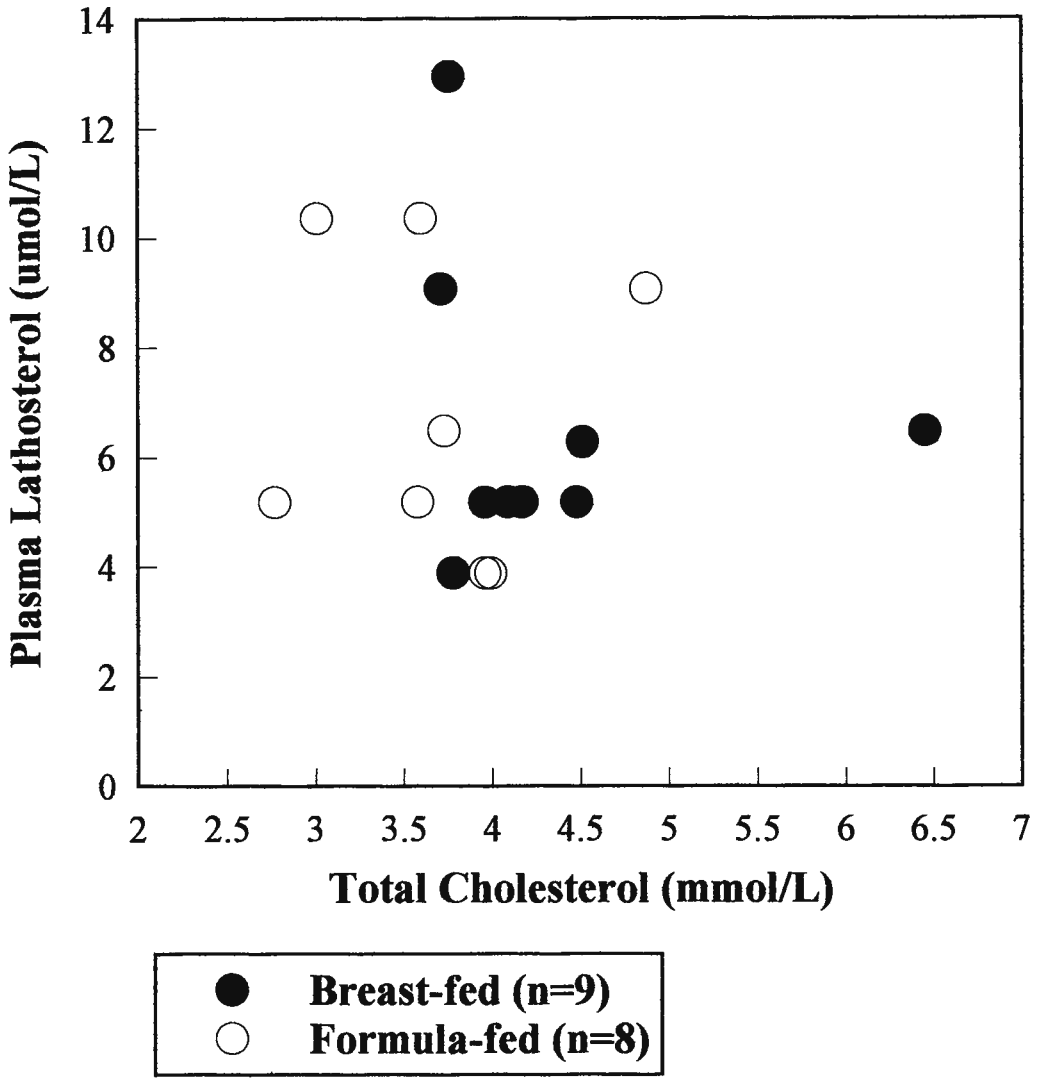
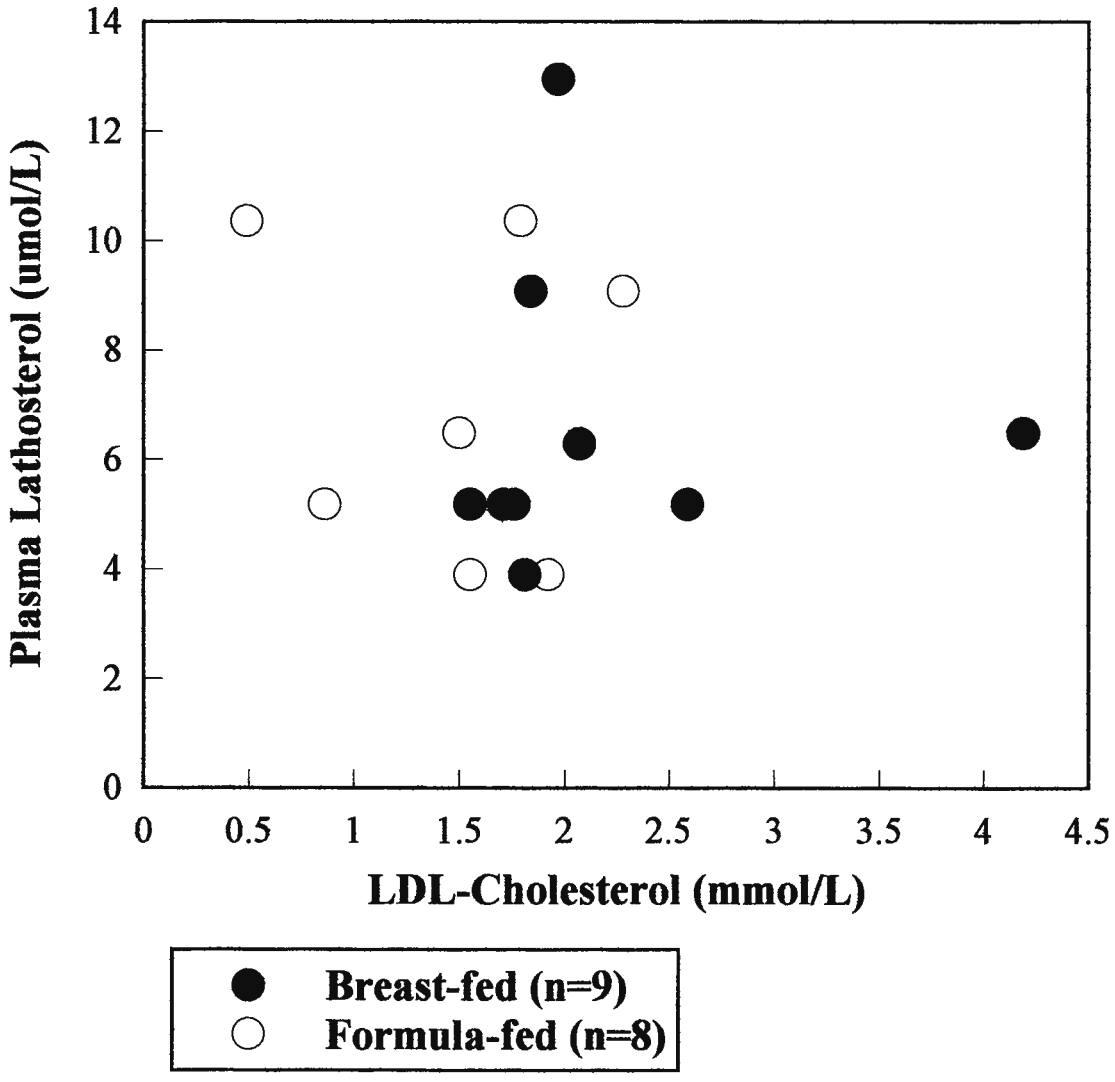


Figure 10 - Plasma lathosterol vs LDL-cholesterol



6. PLASMA LIPIDS OF MALE AND FEMALE BREAST-FED INFANTS

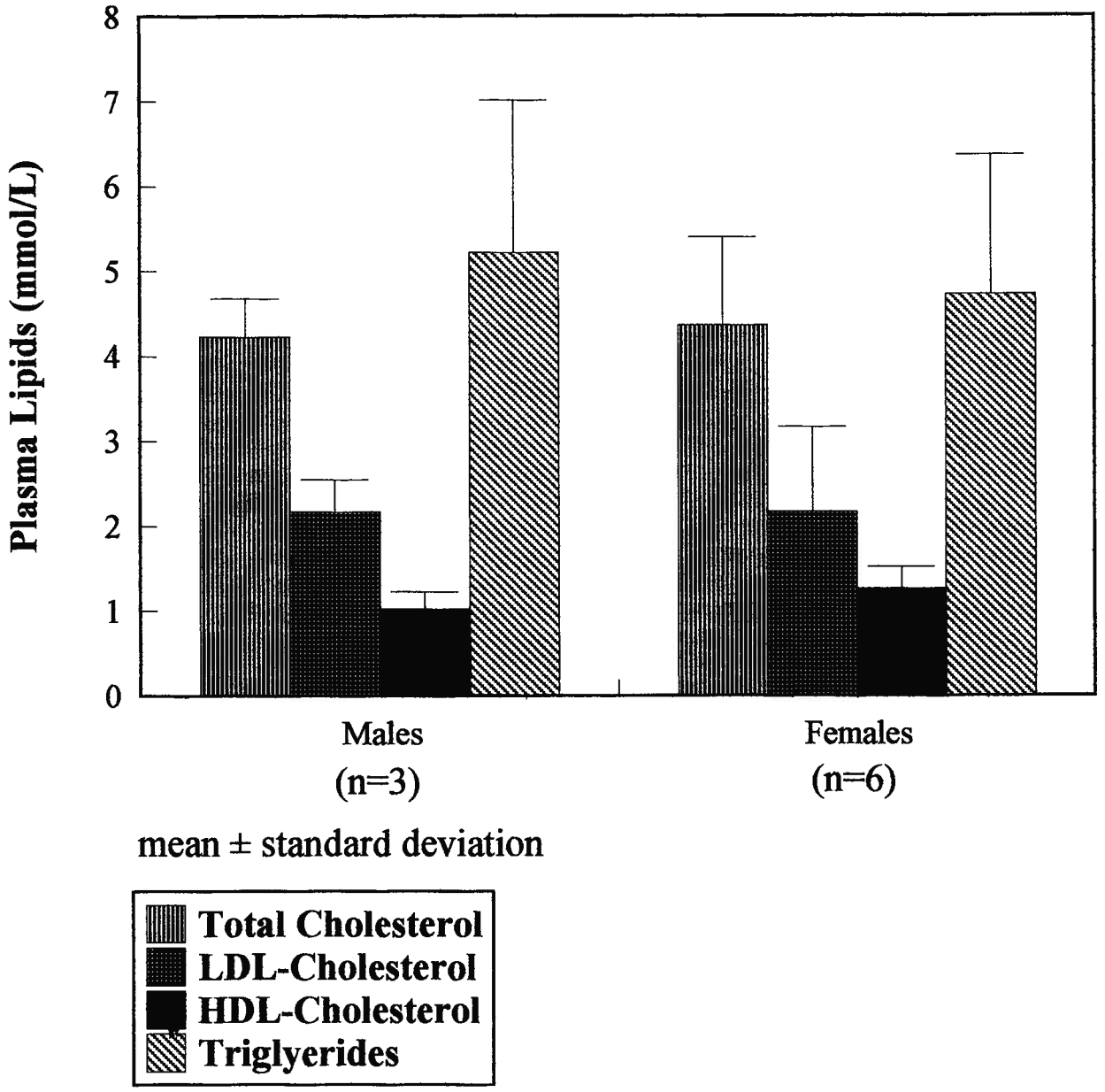
There were no significant ($p=0.839$, 0.998, 0.217 and 0.696 respectively) differences for any of plasma total-cholesterol, LDL-cholesterol, HDL-cholesterol, and triglyceride values between males and females of breast-fed infants - see Table 5 and Figure 11. Analyses of sex differences for the formula-fed group was not carried out, as the modified formula-fed group were all males, and the regular formula-fed group had only one male and two females, thus rendering statistical analysis difficult and of questionable use.

TABLE 5 - Plasma lipids (mmol/L) of male and female breast-fed infants at 4 months

SEX	TOTAL-C	LDL-C	HDL-C	TG
Males:	4.48	2.59	1.22	3.39
	4.51	2.07	1.04	6.97
	3.71	1.84	0.80	5.29
Mean:	4.23 ± 0.45	2.17 ± 0.38	1.02 ± 0.21	5.22 ± 1.79
Females:	3.96	1.55	1.48	4.72
	4.09	1.71	1.24	5.65
	3.76	1.97	1.24	2.69
	4.17	1.76	1.00	7.12
	6.45	4.19	1.63	3.11
	3.78	1.81	0.96	5.10
Mean	**4.37 ± 1.03**	**2.17 ± 1.00**	**1.26 ± 0.26**	**4.73 ± 1.64**

Mean ± Standard Deviation
Total-C = total cholesterol
LDL-C = low density lipoprotein cholesterol
HDL-C = high density lipoprotein cholesterol
TG = triglyercides

FIGURE 11 - Plasma lipids of breast-fed males and females at 4 months



7. CHOLESTEROL SYNTHESIS OF MALE AND FEMALE BREAST-FED INFANTS

There was a significant ($p=0.018$) difference in FSR of male and female breast-fed infants. Males had an average FSR of 3.25±0.68%, whereas females had an average FSR of 1.44±0.92% - see Table 6 and Figure 12.

There was no significant ($p=1.0$) difference in plasma lathosterol or plasma lathosterol:cholesterol between males and females in breast-fed infants - see Table 6 and Figure 13.

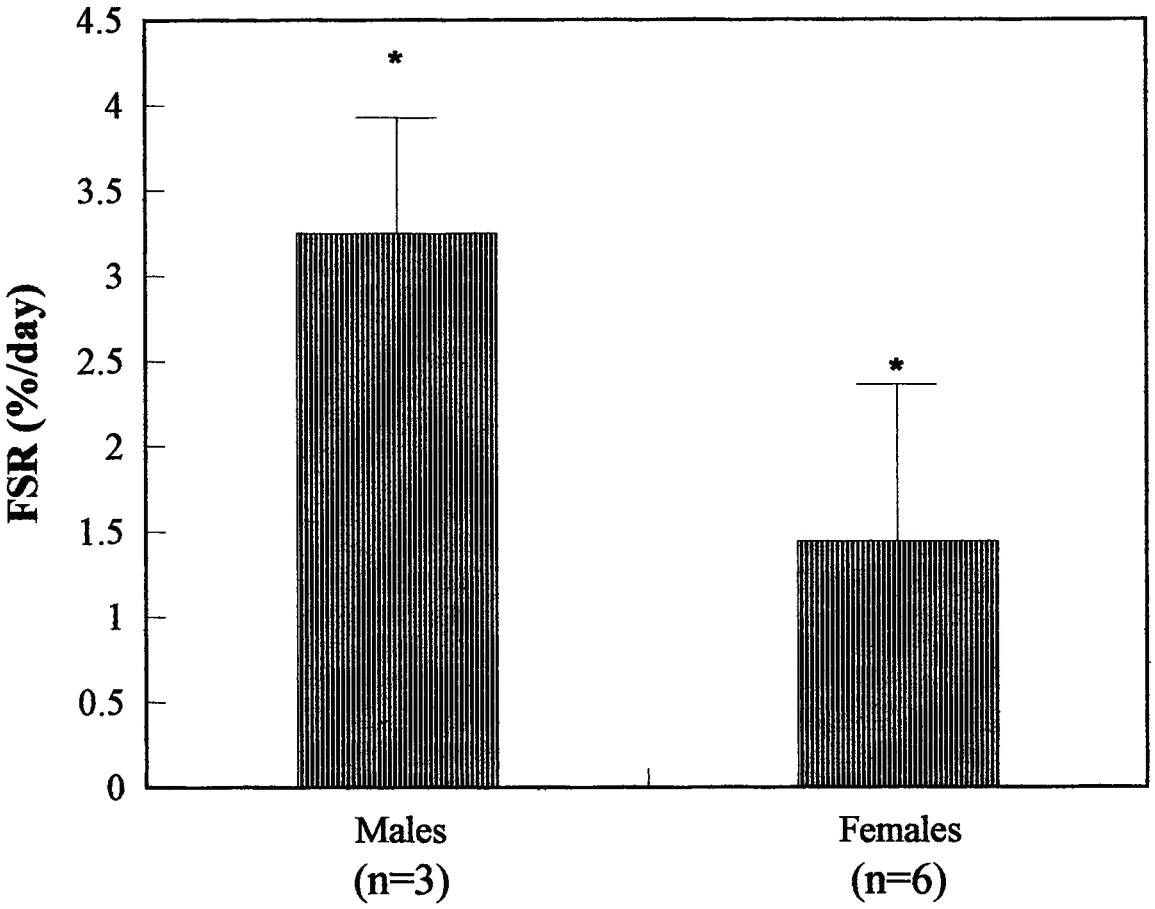
Analyses of sex differences for the formula-fed groups were not carried out, as the modified formula-fed group were all males, and the regular formula-fed group had only one male and two females. This made statistical analysis difficult and not particularly useful.

TABLE 6 - Cholesterol synthesis of male and female breast-fed infants at 4 months

SEX	FSR	PLASMA L:C ($\times 10^{-2}$)	PLASMA LATHOSTEROL µg/200µL
Males:	3.96	0.342	0.004
	2.61	0.443	0.005
	3.17	0.241	0.007
Mean:	**3.25 ± 0.68**	**0.342 ± 0.101**	**0.005 ± 0.002**
CV		0.295	0.286
Females:	0.00	0.140	0.004
	2.16	0.389	0.004
	0.67	0.443	0.010
	1.89	0.264	0.004
	1.52	0.296	0.005
	2.37	0.101	0.003
Mean:	**1.44 ± 0.92**	**0.272 ± 0.134**	**0.005 ± 0.003**
		0.494	0.506

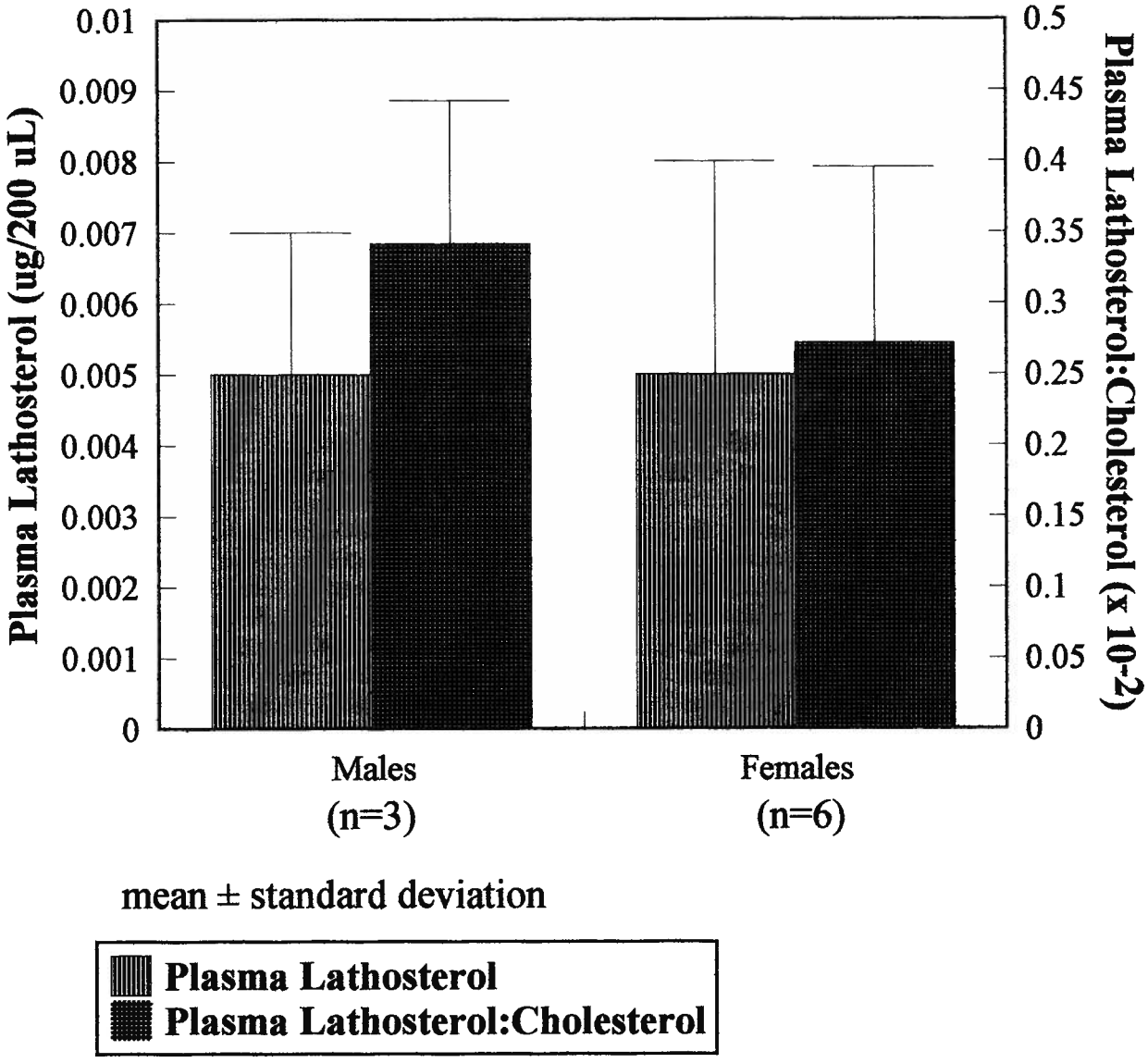
Mean ± standard deviation
FSR of males vs females p = 0.018
FSR = fractional synthetic rate of cholesterol
L:C = lathosterol to cholesterol ratio
CV = coefficient of variation

FIGURE 12 - FSR of breast-fed males and females at 4 months



FSR = fractional synthetic rate of cholesterol
mean ± standard deviation
* p = 0.018

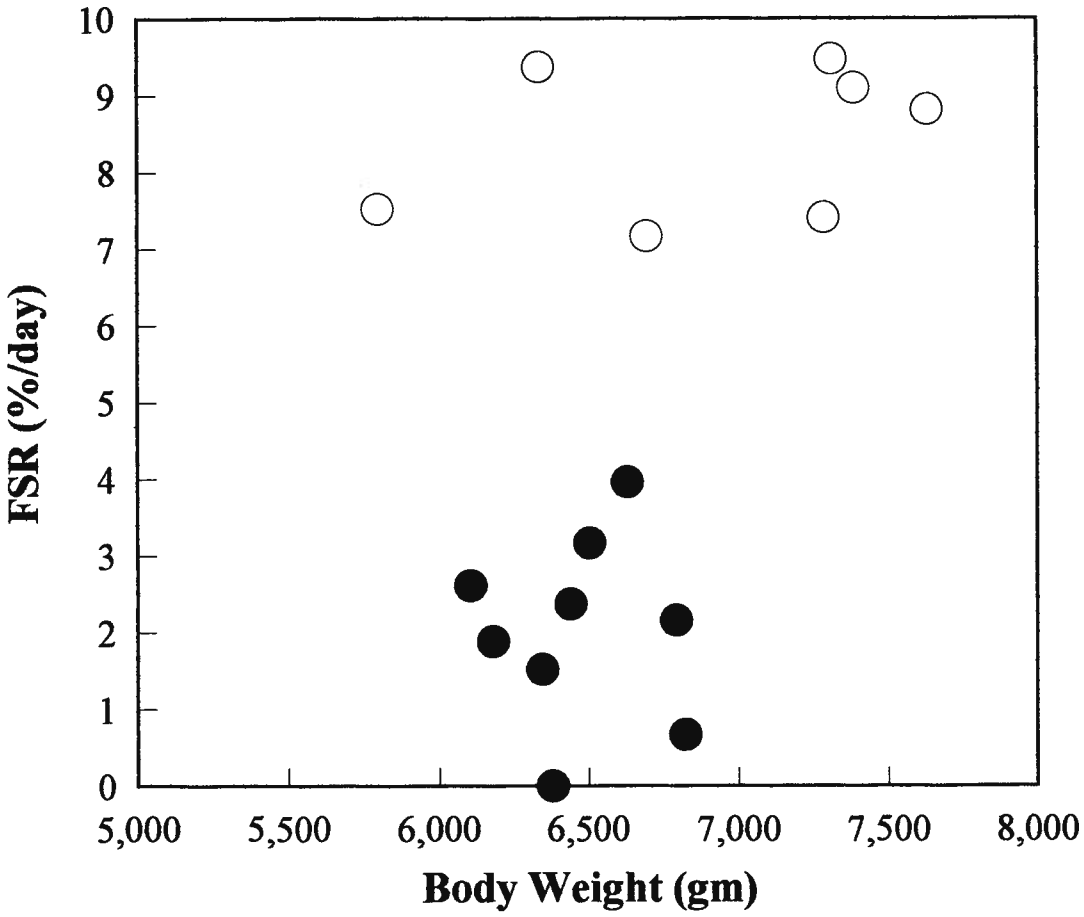
FIGURE 13 - Plasma lathosterol and plasma lathosterol: cholesterol of breast-fed males and females at 4 months



8. RELATIONSHIP OF BODY WEIGHT TO CHOLESTEROL SYNTHESIS

There was no significant ($p=0.920$) relationship between FSR and body weight of the breast-fed and two formula-fed groups as a whole - see Figure 14. There was also no significant ($p=0.182$) relationship between plasma lathosterol and body weight of the breast-fed and two formula-fed groups as a whole - see Figure 15.

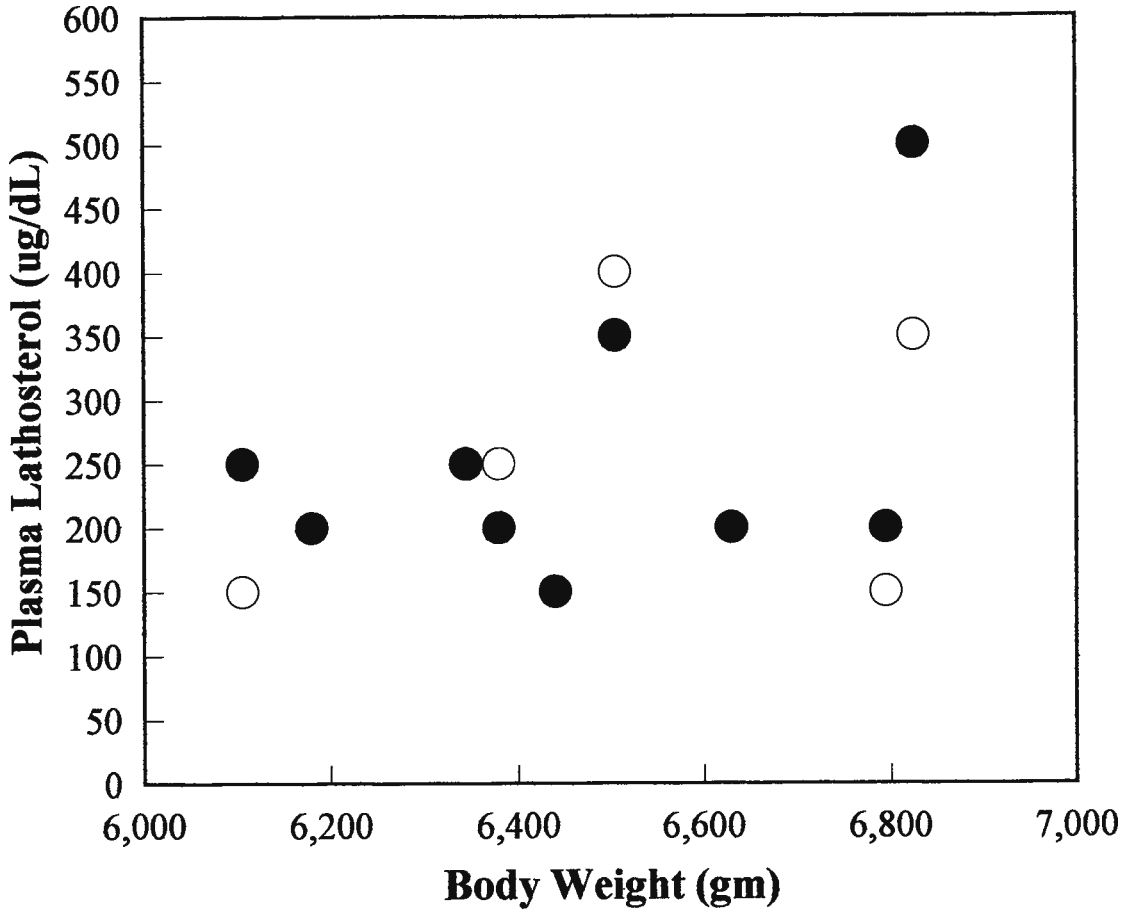
FIGURE 14 - FSR vs body weight* of breast-fed and formula-fed infants at 4 months



FSR = fractional synthetic rate of cholesterol
* Data available for only 7 formula-fed infants

● **Breast-fed (n=9)**
○ **Formula-fed (n=8)**

FIGURE 15 - Plasma lathosterol vs body weight*of breast-fed and formula-fed infants at 4 months



*Data available for only 7 formula-fed infants

● **Breast-fed (n=9)**
○ **Formula-fed (n=8)**

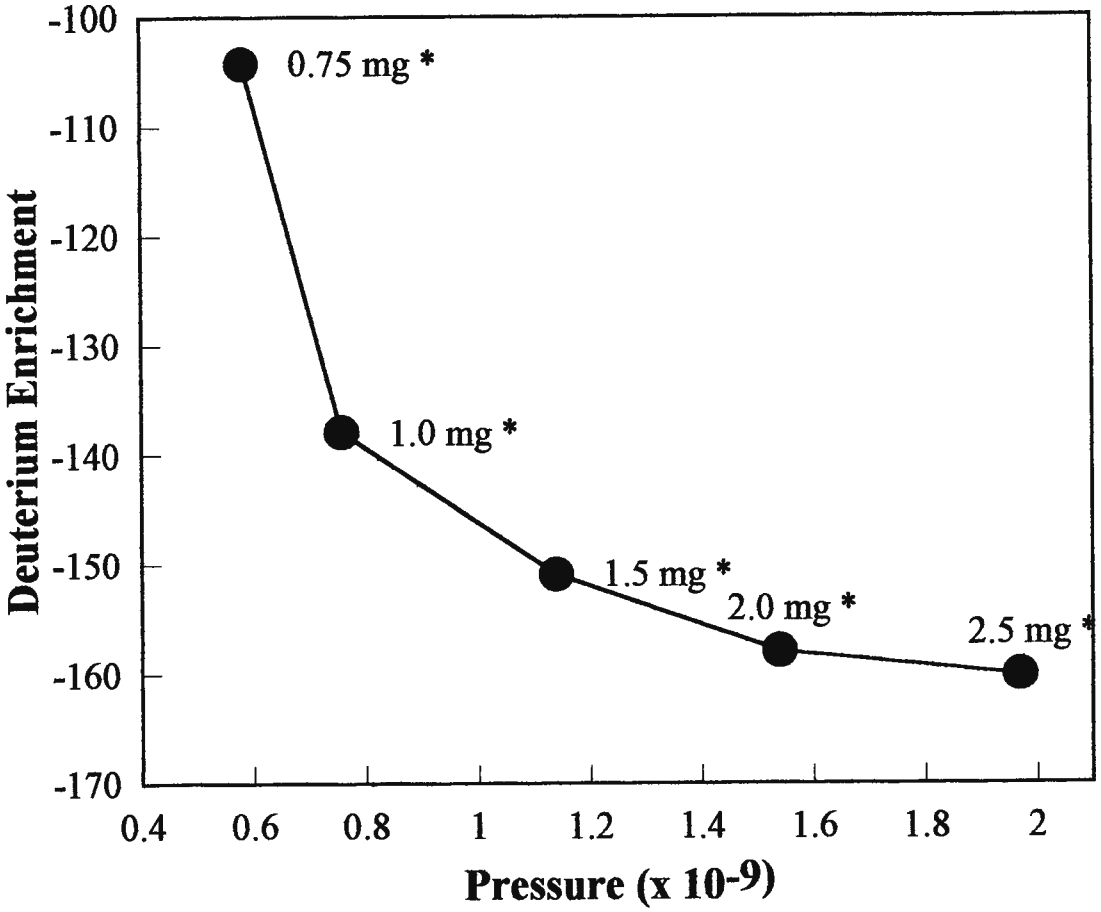
9. BLOOD SAMPLE SIZES

Figure 16 demonstrates a consistent deuterium enrichment for cholesterol standards of 2 mg or more. Lesser amounts, however, began to produce a distortion of the D20/H20 ratio, to reflect a lower deuterium enrichment. The relationship seen for lower amounts of cholesterol appears not to be linear, but rather exponential. Regression analysis was carried out and an r^2 value of 0.881 ($p<.001$) obtained, indicating a strong relationship between the two factors.

These data could be used in experiments supplying only small blood samples. By taking a pressure measurement for each sample run through the mass spectrometer, the amount of cholesterol present could be determined. If the amount of cholesterol is less than 2.0 mg, i.e. beyond the flat portion of the line, the difference in deuterium enrichment could be accounted for and factored into the calculation of cholesterol synthesis.

The equation of the curve of deuterium enrichment versus pressure was determined as y=-185.351 + 41.867(1/x), where y=deuterium enrichment and x=pressure (x10-9). The pressure measurement of a blood sample could be replaced for x and a deuterium enrichment value obtained (a). The same calculation would be carried out for a 2 mg cholesterol sample (b), as this amount exists on the flat part of the line. By dividing b by a, and multiplying this by the original deuterium enrichment value obtained for the blood sample, a new corrected value would be obtained (Note: as mass spectrometer readings fluctuate from one day to another, a new 2 mg cholesterol sample should be run with each new set of blood samples analyzed to obtain a baseline pressure reading for that day). See Appendix 2 for an example.

Figure 16 - Deuterium enrichment vs pressure



* = cholesterol

V DISCUSSION

1. SUBJECT CHARACTERISTICS

The present study looked at differences in specific parameters of cholesterol metabolism between breast-fed and formula-fed infants at four months of age. Sample and subject size presented somewhat of a limitation and must be kept in mind when discussing the results.

The initial experimental design did intend to produce three homogeneous groups of infants, each containing an equal number of males and females, and an equal number of blacks and whites. Difficulties in subject recruitment, however, failed to allow for an even distribution of subject characteristics between the groups. Despite the incentives of free diaper coupons, free formula for the formula-fed infants, as well as free baby food coupons upon completion of the study, mothers were somewhat reluctant to enrol their child into the study. Of the mothers approached, only a small percentage agreed to take the information home with them to discuss with their husbands and further consider enrolling in the study. Of these, only a small fraction ultimately decided to participate. The most probable explanation for the lack of interest was the requirement for the three-day blood draw from each infants. Unless the parents had a keen interest in the subject, or were scientifically inclined, allowing their child to undergo repeated attempts at blood draws was not something they were apt to agree on. Not only did this prove frustrating, but the potential for introducing bias into the study became a concern. It is possible that more highly educated families might be more likely to enrol, based on the observations above. Whether or not this could play a role in cholesterol metabolism at such a young age (four months) is unknown. It is possible that parent-infant interactions could be affected and possibly have an impact on digestive processes and ultimately cholesterol synthesis.

It was difficult to recruit black breast-fed infants. Black mothers were generally of lower income and therefore were eligible for participation in a social assistance program called WIC (Woman, Infant, Child), a common form of assistance in the United States. This program provided free formula for the mothers. As cost was no longer a concern to them, the incentive to breast-feed to save money was low. As well, this population tended to return to work earlier, so that even if breast feeding was initiated, it was not carried on for the full four month period required for the participation in the study. In addition to these problems, breast-feeding was often discouraged in this group. As drug abuse was more common among this population, the risk of passing drugs on to the infant through the breast milk was a concern eliminated by feeding formula.

Seventeen infants had reached four months of age by the time of analysis. The breast-fed group contained nine infants made up of three males and six females, all of whom were white. The formula-fed group contained eight infants. Seven subjects in this group were white, and one was black. A nine-eight split of breast-fed to formula-fed infants would not appear to pose major analysis problems; however, the concern arises in the uneven split of formula-fed infants to either the regular formula or the modified formula. For this there was a three-five split respectively. The modified formula-fed group were all white males. The regular formula-fed group not only contained a low subject number, but in addition, it was made up of one white male, one black male, and one black female. Nonetheless, it is possible that any potential intervening variables of one subject would be cancelled out by intervening variables of the other. In addition, one should keep in mind that the main purpose of the experiment was to determine if the addition of cholesterol to formula could produce similar physiologic effects to those of the breast-fed group. In that respect, the group of five modified formula-fed infants would appear to be able to answer

this question. The group of three regular formula-fed infants, although not a homogeneous group, would provide some evidence for differences seen due to absence of cholesterol in the formula.

After recruitment of infants, the study progressed relatively smoothly. The next problem, however, arose at the time of blood draws. A three-day consecutive blood draw was necessary in order to measure deuterium incorporation into cholesterol, in order to estimate cholesterol synthetic rate. This required substantial commitment on the part of the mother. As infants have quite a significant layer of subcutaneous fat insulating their bodies, location of a vein to obtain blood from was somewhat challenging for the attending nurse. It also posed stressful for some infants and mothers.

The back of the hand or the forearm was used, however, only two attempts in total were allowed, as specified in the ethics section of the research proposal. If no blood was obtained after two insertions of the needle, no sample could be taken. This occurred on three different occasions. Of the infants from whom blood was successfully drawn, the majority of the draws produced very small samples. Ideally, for ease and accuracy of laboratory analysis, a minimum of 1.5 mL of red blood cells in triplicate was necessary. The most ever obtained was a duplicate of 1.5 mL, however, this was the exception. In most cases, only 1 mL was obtained, and in some cases, only a single sample of 1 mL.

The above problems encountered could partially explain why there are virtually no infant studies on this subject. An animal study may have been less expensive and produced fewer complications. A human study was chosen, however, as results do not need to be extrapolated from one species to another.

2. WEIGHT GAIN OF BREAST-FED AND FORMULA-FED INFANTS

A significant difference in weight gain existed between breast-fed and formula-fed infants at four months. More importantly, however, when taken as a percentage weight gain, there was no difference present. This latter measurement is more meaningful, as it measures weight gain relative to birth weight, rather than as an absolute value.

It is generally thought that formula-fed infants gain weight more rapidly than breast-fed infants (Dewey *et al.*, 1993). The supporting data for this, however, is generally based on measurements taken between seven and 24 months of age. As weight gain of the infants in the present study were measured at four months, results cannot be directly compared. In a study of one month-old infants, no differences in weight gain were found between breast-fed and formula-fed infants (Sanchez-Pozo *et al.*, 1986), agreeing with the results of the present study.

As well as age of measurement having an effect on weight gain, the protein content of the diet plays a role. It is known that whey proteins have a high nutritive value and that infants consuming a whey dominant formula, as opposed to a casein dominant formula, grow faster (Jarvenpaa *et al.*, 1982). The formula used in the present study consisted of a 60:40 whey to casein protein blend, as is that of breast milk, unlike the more common 82:18 ratio found in most other cow's milk-based formulas. This also provides some explanation as to why no difference in weight gain was seen between the formula-fed and breast-fed infants in the present study.

3. SERUM LIPIDS OF BREAST-FED AND FORMULA-FED INFANTS

The observation of no difference in serum lipids between breast-fed and formula-fed infants is in contrast to previous findings showing formula-fed infants to have lower serum cholesterol levels than breast-fed infants (Cruz *et al.*, 1992; Huttunen *et al.*, 1983; Mellies *et al.*, 1978; Friedman and Goldberg, 1975). This belief is also supported by similar results demonstrated in animals (Jones *et al.*, 1990; Mott *et al.*, 1982; Kris-Etherton *et al.*, 1979).

The high cholesterol content of breast milk is expected to produce higher serum total- and LDL-cholesterol levels than when formula is used. Indeed this was found in the study by Cruz *et al.* (1992). Total- and LDL-cholesterol levels for the breast-fed group were 4.35±0.18 and 2.44±0.23 mmol/L respectively, versus 3.32±0.28 and 1.42±0.31 mmol/L for the cow milk-based formula group, 3.08±0.32 and 1.01±0.31 mmol/L for the soy-based formula group, and 3.42±0.18 and 1.50±0.21 mmol/L for the modified soy-based formula group.

In addition, there was an inverse relationship between FSR and total- and LDL-cholesterol for all groups combined. Similar results were observed by Wong *et al.* (1993) who used three different types of formula. The combined mean was 2.90±0.57 and 1.24±0.41 mmol/L for total- and LDL-cholesterol respectively for the formula-fed groups, and 4.74±1.22 and 2.15±0.67 mmol/L for the breast fed group.

Why such results were not observed in the present study is not certain. One could dismiss the results as being due to inadequate sample numbers; however, there are other not so obvious differences present between breast milk and infant formulas, as to be discussed later.

4. CHOLESTEROL SYNTHESIS AS MEASURED BY DEUTERIUM ENRICHMENT

Cholesterol synthetic rates, as measured by deuterium uptake into red blood cells, were lower in the breast-fed infants than the formula-fed infants. These findings are in agreement with previous research of male infants by Cruz *et al.* (1992), showing breast-fed infants to have a significantly lower FSR than formula-fed infants at four months. Breast-fed infants (n=12) in this study had an FSR of 2.62% per day, similar to that found in the present study. Three different types of formula were used in the study by Cruz. Cow's milk-based (1.1 mg cholesterol/dL) formula-fed infants (n=8) had an FSR of 6.90% per day. Soy-based (0 mg cholesterol/dL) formula-fed infants (n=7) had an FSR of 8.03% per day. The addition of 1 mg cholesterol/dL to the soy-based formula, to match that present in the cow milk-based formula, resulted in a moderate lowering of FSR in another group of infants (n=6). One might conclude therefore, that the addition of cholesterol to milk formula results in a lowering of FSR. Further, one could speculate that the addition of more cholesterol, to match the amount found in breast milk, would lower the FSR even further. The present study, however, does not support this hypothesis. In fact the addition of cholesterol to the formula resulted in a slightly higher, nonsignificant FSR (8.53% per day versus 8.29% per day). Why the addition of cholesterol to formula in previous research has produced an effect may be explained by the types of formula used. The cow milk-based formula may not have been as similar in composition to breast milk as was the cow milk-based formula used in the present research. Further, soy-based formulas are even less similar in composition to breast milk, specifically in the protein and carbohydrate present.

Soy protein has been shown to have a hypocholesterolemic and antiatherogenic effect in humans (Carroll, 1991), when compared with casein, the major protein of cow's milk. In fact, the response may be greater in younger than in older subjects. Further, the addition of cholesterol to

the diet (500 mg/day) results in a more marked effect than is seen on a lower cholesterol diet (100 mg/day) (Meinertz *et al.*, 1990).

Perhaps the addition of cholesterol to soy formula resulted in a greater compositional similarity to that of cow milk-based formula, thus producing a more similar FSR. One can only speculate what may have happened if cholesterol was added to physiologic amounts of that found in breast milk. Since addition of cholesterol to formula in the present study appears to have no impact on cholesterol synthesis, one must look at other compositional differences between breast milk and the formula used. The present study used a cow milk-based formula which was the most similar in nutritional composition to breast milk, of all the commercially available infant formulas on the market. The carbohydrate (CHO) in breast milk is exclusively lactose, as was the formula. There were 7.2 gm CHO/dL in both. The protein content of breast milk is 1.05±0.2 gm/dL. The formula contained 1.5 gm/dL. The type of protein present in the formula was a 60:40 whey to casein blend, which is identical to that found in breast milk. Total fat content of mature human milk is 3.9±0.4 gm/dL, compared to 3.6 gm/dL found in the formula.

Micronutrient composition is virtually identical between breast milk and the formula used. With regard to macronutrients, the only difference in protein and CHO composition between milk and the formula used is that one originates from human milk, whereas the other comes from bovine milk. The fat blend of the formula was made up of various vegetable oils. Possible explanations for the results observed will be expanded upon later in the discussion.

5. CHOLESTEROL SYNTHESIS AS MEASURED BY SERUM LATHOSTEROL

The marked difference in cholesterol synthesis observed between breast-fed and formula-fed infants, as measured by deuterium incorporation methodology, was not seen when measured by lathosterol analysis. Lathosterol, an immediate precursor of cholesterol, would be expected to be high during times of increased cholesterol synthesis, i.e. in the formula-fed infant. Studies in adults have shown serum lathosterol to be a reliable measure of cholesterol synthesis. For example, Miettinen *et al.* (1990), showed lathosterol, in terms of ug/mg cholesterol, to be positively related to overall cholesterol synthesis in adult males. This finding has been supported by other research as well (Kempen *et al.*, 1988; Bjorkhem *et al.*, 1987). In addition, a study of pre-term infants using lathosterol analysis, has shown it to be a useful measure of cholesterol synthesis (Hamilton and Phang *et al.*, 1992).

A study of term infants in the first four days of life, however, showed no difference in plasma lathosterol levels between breast-fed and formula-fed infants (Hamilton and Synnes *et al.*, 1992). One might interpret this as meaning there was no difference in cholesterol synthesis between the two groups, despite higher serum cholesterol levels seen in the formula-fed infants. On the other hand, it could provide support for the observations seen in the present study. That is, differences in cholesterol synthesis may indeed exist if measured by deuterium incorporation methodology, although differences in serum lathosterol are not observed.

The question arises as to why lathosterol analysis is a reliable measure of cholesterol synthesis in adults and pre-term infants, but not in term infants. The only obvious difference between these groups is the diet. The pre-term infants described earlier were formula-fed, whereas adults consume a varied diet. This will present some differences from breast milk. Since breast milk is high in cholesterol, reflecting a high rate of cholesterol synthesis in the mammary gland (Kallio *et*

al., 1989), it is feasible that it would also be significantly high in its biosynthetic precursor, lathosterol. This would be passed on to the infant through feeding, as is cholesterol, and produce higher serum lathosterol levels in breast-fed infants, despite low levels of cholesterol synthesis in the breast-fed infant. This could be responsible for the lack of difference seen between breast-fed and formula-fed infants in the present study.

At two months lactation, the lathosterol concentration of breast milk is approximately 1.1±0.43 umol/L and rises to 2.3 umol/L by 6 months (Kallio *et al.*, 1989). Plasma lathosterol levels of breast-fed infants at four months in the present study were on average, 6.62 umol/L. This represents a small enough concentration to be affected by the small quantities present in breast milk.

6. RELATIONSHIP OF FSR TO PLASMA LIPIDS

In the study by Cruz *et al.* (1992), those infants with low serum total- and LDL-cholesterol had high rates of cholesterol synthesis. This inverse relationship of all groups combined began to emerge in the present study, but did not reach statistical significance ($p=0.069$). Again, these results are puzzling, as was previously discussed regarding serum lipids. Perhaps a larger study group would have produced a significant difference.

Garbagnati (1993) found that fever in children, no matter what the cause, may be associated with a decrease in serum cholesterol. Whereas this situation was noted in one subject in the present study, no control for this aspect was made. Further, it is unlikely that the situation would have repeated itself in enough infants to be responsible for the results seen. Other possibilities will be elaborated upon later in the discussion.

7. FAT BLEND OF BREAST MILK AND INFANT FORMULA

As cholesterol did not appear to be having any effect on cholesterol synthesis and serum lipids, the next most logical explanation for the differences observed in breast-fed and formula-fed infants would be variations in the fat blend. The cholesterol-raising ability of saturated fats (SFA) as opposed to monounsaturated fats (MUFA) and polyunsaturated fat (PUFA), has been well established (McNamara, 1987). The fat blend of the formula used in the present study was very similar to that of breast milk, with regard to the PUFA:SFA (P:S) ratio (P:S=0.32:1 for breast milk versus 0.33:1 for the formula). Further examination of the precise fatty acids present, however, reveals some significant differences - see Appendix 4. Mature human milk contains approximately 4.4% lauric acid (C12:0) and approximately 23% palmitic acid (C16:0) (Lammi-Keefe and Jensen, 1984). The milk formula used, however, contained 11.9% C12:0 and 13.8% C16:0. Lauric acid has been shown to have a cholesterol raising effect (Denke and Grundy, 1992). Consequently, the higher concentration of lauric acid in the formula would be expected to result in higher concentrations of plasma total- and LDL-cholesterol. One may speculate that this is due to an effect of cholesterol synthesis. This may bring the values closer to those observed in breast-fed infants, thus providing some explanation for the lack of significant difference between the two.

On the other hand, palmitic acid, which has an even greater cholesterol-raising effect than lauric acid (Denke and Grundy, 1992), is present in lower concentrations in the formula than in breast milk. This would be expected to result in the opposite effect, i.e. lower concentrations of plasma total- and LDL-cholesterol. In this respect a lower FSR would be expected. Thus, it appears that the effects of C12:0 would cancel the effects of C16:0. It is interesting to note, however, that C12:0 is more readily absorbed than C16:0, both as an individual fatty acid, and

when esterified in the sn-2 position (Tomarelli *et al.*, 1968). This could result in higher concentrations of plasma total- and LDL-cholesterol after all, as the C12:0 would be more readily available to exert an effect.

8. POSITIONAL DISTRIBUTION OF THE FATTY ACIDS IN BREAST MILK AND INFANT MILK FORMULA

Although the P:S ratios are very similar between breast milk and the formula used, some positional differences between the fats used do exist. That is, where the fatty acid exists on the triglyceride molecule may be playing a role, as this is important in fat digestion, absorption, and metabolism. In fact, location of C16:0 in human milk in the sn-2 position enhances absorption of dietary fats (Tomarelli *et al.*, 1968; Jensen *et al.*, 1982). Pancreatic lipase specifically attacks the primary ester linkages (Mattson *et al.*, 1952). Digestion of triglycerides by lipase hydrolysis yields sn-2 monoglycerides and free fatty acids released from the sn-1,3 positions (Small, 1992). This renders a large proportion of the palmitic acid present in the intestinal tract as a monoglyceride. Monoglycerides are believed to be better absorbed than their respective free fatty acids.

In human milk triglycerides, approximately 70% of the C16:0 is located in the center sn-2 position of the triglyceride molecule (Jensen, 1989), whereas in bovine milk, it is split between the sn-1 and sn-2 positions (Tomarelli *et al.*, 1968). In fact, in vegetable and animal tissue lipids, with the exception of certain animal fats, more than 80% of the C16:0 is esterified to the sn-1,3 carbons on the triglyceride molecule (Parodi, 1982). This represents a significant difference between infant formula fats and those found in breast milk. In fact, the fat blend of the formula used in the present study consisted of a mixture of oleo, coconut, soybean, and oleic (sunflower

or safflower) oils. The proportion of sn-2 C16:0 is low in oleo, soybean, and coconut oils, and sunflower and safflower contain no C16:0 (Tomarelli *et al.*, 1969). Therefore, absorption of palmitic acid (C16:0) would be expected to be greater from human milk than from infant formula, which contains fat with C16:0 esterified predominantly in the sn-1,3 positions (Lammi-Keefe and Jensen, 1984). This provides reasonable evidence for a cholesterol synthesis suppressing effect of human milk versus formula, despite similarities in cholesterol content.

The only sufficient way of confirming the absence of an effect of cholesterol on FSR in infants would be to compare one breast-fed group with another breast-fed group in which the cholesterol has been removed from the milk. The impracticality of this makes it virtually impossible. Another solution may be to feed lactating mothers a cholesterol-lowering diet with administration of cholestyramine or lovastaton. There may be some concern, however, regarding the possibility of drug metabolites in the milk being passed on to the infants. Further, it is questionable whether drug effects on cholesterol metabolism in the liver would have the same effect on that occurring in the mammary gland.

9. TYPE OF CHOLESTEROL AND CHOLESTEROL SYNTHESIS

The type of cholesterol added to the modified formula was free cholesterol, as opposed to esterified cholesterol. One might assume that free cholesterol would be readily absorbed and expected to exert a pronounced effect in the body. Breast milk however, contains 16.5-24.0% esterified cholesterol as opposed to free cholesterol, depending on the stage of lactation (Lammi-Keefe and Jensen, 1984).

The major fatty acids esterified with cholesterol are C16:0, C18:1, and C18:2. Milk cholesterol esters are found in the fat globules and aqueous phase of milk (Lammi-Keefe and

Jensen, 1984), whereas free cholesterol would be expected to exist in the aqueous phase only. Given the effects of C16:0 on fat absorption and digestion previously described, one may speculate that esterified cholesterol would exert a greater effect on lipid metabolism than would free cholesterol. Addition of free cholesterol to infant formula may be hypothesized to elevate circulating cholesterol levels. In fact, infants fed cholesterol supplemented milk formulas have been shown to have similar cholesterol levels, as compared to those fed breast milk (Friedman and Goldberg, 1975). This cholesterol may not cause feedback inhibition of cholesterol synthesis as would esterified cholesterol.

10. OTHER STEROLS IN HUMAN MILK AND POSSIBLE EFFECTS ON CHOLESTEROL METABOLISM

The phytosterol content of human milk ranges from 90-300 mg/100 gm fat (Lammi-Keefe, 1984). It has been suggested that individual phytosterols may possibly be having effects on sterol absorption and transfer, but whether such effects would be seen with the amounts present in breast milk is not known. Phytosterol content of the infant formula is a factor which was not controlled for in the present study. If the phytosterol in human milk is acting to suppress cholesterol synthesis, this could explain why the formula-fed infants did not suppress cholesterol synthesis, as infant formula does not contain any phytosterols.

Desmosterol, the immediate precursor of cholesterol in the biosynthetic pathway, is present in human milk in small quantities, 0.6 mg/100 mL milk at two weeks to 1.3 mg/100 mL milk at 16 weeks (Lammi-Keefe and Jensen, 1984). Desmosterol makes up approximately 10% of the total

sterols in human milk (Clark *et al.*, 1983). It's role is not known, but one may consider a potential effect on cholesterol metabolism. There are no studies examining this possibility.

11. PROTEIN AND ITS EFFECTS ON CHOLESTEROL METABOLISM

The similarity in serum lipids between the breast-fed and formula-fed infants may be due to something other than the addition of cholesterol to the infant formula. The formula used in the present study contains a 60:40 whey to casein protein blend, unlike the more typical 82:18 ratio found in most other adapted cow's milk-based formulas.

High casein containing diets have been reported to result in higher serum total- and LDL-cholesterol levels than lower casein containing diets (Kurowska and Carroll, 1990). This effect appears to be associated with a decrease in receptor-mediated LDL catabolism (Carroll, 1992), as well as impaired receptor-dependent removal of LDL-apo B (Samman *et al.*, 1990). Further, it has been reported that infants fed whey predominant formulas have higher serum cholesterol levels than those fed a casein predominant formula (Jarvenpaa *et al.*, 1982). This would bring serum cholesterol levels closer to that of a breast-fed infant, and potentially result in no significant differences between the two, as was observed in the present study. The mechanism by which this is occurring is not known.

12. NUCLEOTIDES AND THEIR EFFECTS ON CHOLESTEROL METABOLISM

Nucleotides, the precursors of DNA and RNA, have been shown to have an impact on cholesterol metabolism in infants (Gil 1983; Sanchez-Pozo, 1986). Breast milk contains significant quantities of nucleotides (Sanchez-Pozo, 1986). Breast-fed babies and babies fed formula with supplemental nucleotides, have higher plasma HDL-cholesterol and lower plasma

VLDL-cholesterol at one month than formula-fed infants alone. Incidentally, plasma levels of long-chain PUFAs are also higher in the two nucleotide-fed groups. One might speculate that the presence of nucleotides in the breast milk is having an impact on cholesterol synthesis, and is responsible for the differences observed with the formula-fed infants. Analysis of the composition of the formula used in this study, however, reveals that it has been fortified with nucleotides - see Appendix 5.

The presence of nucleotides provides some explanation as to the similarity in serum lipid values seen in the breast-fed and formula-fed infants. Further, it may be overriding an effect of cholesterol fortification of infant formula, which would explain the similarity in serum lipid values seen between the two formula-fed groups.

Dietary nucleotides may influence liver or enterocyte lipid synthesis by stimulating apolipid formation (Sanchez-Pozo *et al.*, 1986). Kubota (1969) found weanling rats to have a higher incorporation of dietary nucleotides into the hepatic RNA and DNA than mature rats. Further, Savaiano and Clifford (1981) showed that dietary nucleotides are used by intestinal cells for DNA and RNA synthesis. Whether nucleotides are having an effect directly on cholesterol synthesis in the same fashion has not been studied.

Research by Cruz *et al.* (1992) used formulas which were not supplemented with nucleotides. Differences in total serum cholesterol and LDL-cholesterol were found between breast-fed and formula-fed infants. The formulas used were cow milk-based, soy-based, and modified soy-based with 1.1 mg/dL added cholesterol. This finding provides further evidence for an effect of nucleotides on serum lipids.

13. CHOLESTEROL SYNTHESIS OF MALE AND FEMALE BREAST- AND FORMULA-FED INFANTS

The significant difference found in cholesterol synthesis, as measured via deuterium incorporation methodology, between breast-fed males and breast-fed females could be no more than coincidence, considering the small subject numbers (i.e. only three males compared with six females). Nonetheless, the lower rate of cholesterol synthesis in females than in males is worth noting. There are no studies examining sex differences in cholesterol synthesis in infants, however, cord blood serum total- and LDL-cholesterol concentrations are higher in female than in male infants (Frerichs *et al.*, 1978) and this difference may continue into childhood and throughout adolescence (Frerichs *et al.*, 1976), It is possible that the higher serum concentrations are negatively feeding back on cholesterol synthesis and resulting in reduced rates of synthesis in the females.

Adult studies point to a protective effect of estrogen against atherosclerosis (Kiel *et al.*, 1989); however, cholesterol synthetic rates have not been elucidated as the factor affected. Changes in plasma lipid and lipid levels have been suggested, but the way in which this occurs is not known. A positive correlation has been found between estradiol and total and HDL-cholesterol (Kiel *et al.*, 1989). As well, a direct vascular action, such as endothelial degradation of LDL-cholesterol has been suggested (Riedel *et al.*, 1993). The present study, however, showed no differences in serum lipids between males and females.

Whereas it is possible that the differences seen with estrogen may be due to lowered cholesterol synthesis, the hormonal differences in infants, although present, are not as pronounced as in adults, and therefore would have questionable effects.

14. SERUM LIPIDS OF BLACKS AND WHITES

There was not a large enough group of black infants to perform statistical analysis in comparison with whites, however, it is interesting to note the differences seen. It is known that cord blood serum total- and LDL-cholesterol concentrations are higher in white compared to black infants (Frerichs *et al.*, 1978). One could speculate that the same differences may be seen in plasma values. The one black breast-fed infant in the present study had a total cholesterol concentration of 3.78 mmol/L and LDL-cholesterol concentration of 1.81 mmol/L. This was indeed lower than the averages for the breast-fed group of 4.33 and 2.18 mmol/L respectively, but not the lowest of the whole group (3.70 and 1.55 mmol/L respectively).

The two black formula-fed infants had total serum cholesterol concentrations of 2.77 and 3.60 mmol/L, which are lower than the 3.68 mmol/L average. LDL-cholesterol concentrations were 0.85 and 1.79 mmol/L, compared to the 1.50 average for the group. It would be interesting to have examined further and possibly confirmed a difference between blacks and whites, had a larger group been recruited.

15. RELATIONSHIP OF BODY WEIGHT TO CHOLESTEROL SYNTHESIS

There was no significant relationship between body weight and FSR. One might speculate that a higher body weight would result in greater absolute synthesis of cholesterol. In fact, the rate of cholesterol synthesis per kilogram of body weight varies significantly with the size of the animal (Dietshy *et al.*, 1993). The differences in body weights in the present study were likely not great enough to exert an effect. Further, FSR is a percentage measurement of the total cholesterol in the blood, rather than an absolute value. Larger bodies would not necessarily contain more synthesized cholesterol in the blood, when measured on a percentage basis.

16. BLOOD SAMPLING SIZES

Appendix 3 shows the original and corrected deuterium values for five of the 17 subjects. For the most part, the final FSR was higher than before the correction factor was taken into account (3-40% higher), however, in one case it was lower (6% lower). One can only speculate if statistical analysis of corrected values would have changed the interpretation of results. The lack of data in the present study prevents such analysis, but remains a useful finding to be used by future researchers.

VI CONCLUSIONS

Previous research has shown breast-fed infants to have a low rate of cholesterol synthesis relative to formula-fed infants. The results of the present study support this finding. It appears, however, that the high cholesterol content of breast milk is not the causative factor, as had been hypothesized. This finding was somewhat perplexing, as the formula used was very similar to breast milk in nutritional composition. In addition, previously observed differences in serum total- and LDL-cholesterol between breast-fed and formula-fed infants were not seen. This latter point may be a result of compositional similarities between the different milks, such as the protein content, the fat blend, or the presence of nucleotides.

Differences in cholesterol synthesis between breast- and formula-fed infants may be due to the location of specific fatty acids on the triglyceride molecule, or due to esterification of cholesterol.. The results of the present study reveal the complexity of the situation and the inability to attribute certain body functions to simple, overt differences in nutrient intake. Future studies will need to examine structural differences in dietary nutrients of infants, and not just absolute quantities.

Whereas the small study group warrants careful interpretation of data, based on the results of this study, it would appear that cholesterol fortification of infant formulas is not beneficial to the infant. Formulation of an infant formula containing triglyceride molecules closer in composition to that found in breast milk is an area of research which deserves some attention. The possibility of providing the formula-fed infant with all of the benefits of breast milk may one day be possible, however, further research and discovery has yet to be achieved.

REFERENCES

Anderson JM and Dietschy JM. Absolute rates of cholesterol synthesis in extrahepatic tissues measured with 3H-labelled water and 14C-labelled substrates. J Lipid Res 20:740-752, 1979

Bekaert ED, Alaupovic P, Knight-Gibson CS, Franceschini G and Sirtori CR. Apolipid A-I: sex-related differences in the concentration and composition of apo A-I and apo B-containing lipid particles. J Lipid Res 34:111-123, 1993

Beynen AC and Katan MB. Effect of egg yolk feeding on the concentration and composition of serum lipids in man. Atherosclerosis 54:157-166, 1985

Bhattacharyya AK and Eggen DA. Cholesterol absorption and turnover in rhesus monkeys as measured by two methods. J Lipid Res 21:518-524, 1980

Bjorkhem I, Miettinen T, Reihner E, Ewerth S, Angelin B, Einarsson K. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. J Lipid Res 28:1137-1143, 1987

Burgos C, Castiool M, Rodruguez-Vico F, Zafra MF and Garcia-Peregrin E. Influence of protein/lipid ratio of diet on cholesterol synthesis and esterification in eel liver. Arch Int Physiol Biochim 101:53-55, 1993

Carroll KK. Dietary protein, cholesterolemia and atherosclerosis. Can Med Assoc J 147:900, 1992

Carroll KK. Review of clinical studies on cholesterol-lowering response to soy protein. J Am Diet Assoc 91:820-827, 1991

Carulli N, Tripodi A and Carubbi F. Assay of HMG-CoA reductase activity in the evaluation of cholesterol synthesis in man. Clinica Chimica Acta 83:77-82, 1989

Christison R. Treatise on poisons; 2nd ed. 62-63 (Black, Edinburgh, 1832)

Clark RM, Fey MB, Jensen RG and Hill DW. Desmosterol in human. Lipids 18:264-266, 1983

Clifton PM, Kestin M, Abbey M, Drysdale M, Nestel PJ. Relationship between sensitivity to dietary fat and dietary cholesterol. Arteriosclerosis 10:394-401, 1990

Connor WE and Lin DS. The intestinal absorption of dietary cholesterol by hypercholesterolemic (Type II) and normocholesterolemic human. J Clin Invest 53:1062-1070, 1974

Cruz MI, Wong WW, Mimouni F, Hachey DL, Klein PD and Tsang RC. Effect of dietary cholesterol on cholesterol synthesis rates in infants. Pediatr Res 31:106A, 1992

Cruz ML, Mimouni F, Wong WW, Hachey DL, Klein PD, Tsang RC. Effect of diet in early infancy on cholesterol synthesis rates at one year of life (unpublished data)

Dell RB, Mott GE, Jackson EM, Ramarkrishan R, Carey KD, McGill HC and Goodman DS. Whole body and tissue cholesterol turnover in the baboon. J Lipid Res 26:327-337, 1985

Denke MA and Grundy SM. Comparison of effects of lauric acid and palmitic acid on plasma lipids and lipids. Am J Clin Nutr 56:895-898, 1992

Dewey KG, Heinig MJ, Nommsen LA, Peerson JM and Lonnerdal B. Breast-fed infants are leaner than formula-fed infants at 1 year of age: the DARLING study. Am J Clin Nutr 57:140-145, 1993

Dietschy JM and Spady DK. Measurement of rates of cholesterol synthesis using tritiated water. J Lipid Res 25:1469-1476, 1984

Dory L, Bhattacharyya A, Strong J and Chappuis C. Hepatic low density lipid receptors, HMG-CoA reductase, and plasma lipids and apolipids in high- and low-responding rhesus monkeys: effect of cholestyramine treatment. J Lipid Res 31:279-287, 1990

Edington J, Geekie M, Carter R et al. Effect of dietary cholesterol on plasma cholesterol concentration in subjects following reduced fat, high fibre diet. Br Med J 294:333-336, 1987

Elcarte Lopez R, Villa Elizaga I, Sada Goni J, Gasco Eguiluz M, Oyarzabal Irigoyen M, Sola Mateos A, Garcia Ibero C, Elcarte Lopez T, Ferrer Gimenez M, Fonteneda Estibaliz A. Study from Navarra. Hyperlipidemia II. Variations according to age and sex in the average cholesterol level, LDL cholesterol, and triglycerides in an infant-child population. Anales Expanoles De Pediatria 38(2):159-166, 1993

Ernst JA, Brady MS and Rickard K. Food and nutrient intake of 6 to 12 month old infants fed formula or cow milk: a summary of four national surveys. J Pediatr 117(Suppl)(Part2):S86-100, 1990

Fernandez ML, Young NY and McNamara D. Whole body and hepatic cholesterol synthesis rates in the guinea-pig: effect of dietary fat quality. Biochim Biophys Acta 1044:340-348, 1990

Fjeld CR, Brown KH and Schoeller DA. Validation of the deuterium oxide method for measuring average daily milk intake in infants. Am J Clin Nutr 48:671-679, 1988

Flynn MA, Nolph GB, Flynn TC, Kahrs R and Krause G. Effect of dietary egg on human serum cholesterol and triglycerides. Am J Clin Nutr 32:1051-1071, 1979

Fomon SJ, Rogers RR, Ziegler EE, Nelson SE and Thomas LN. Indices of fatness and serum cholesterol at age eight years in relation to feeding and growth during early infancy. Pediatr Res 18:1233-1238, 1984

Frerichs RR, Srinivasan SR, Webber LS, Rieth MC and Berenson GS. Serum lipids and lipids at birth in a biracial population: The Bogalusa heart study. Pediatr Res 12:858-863, 1978

Frerichs RR et al. Serum cholesterol and triglyceride levels in 3,446 children from a biracial community - The bogalusa heart study 54:302-309, 1976

Friedman G and Goldberg SJ. Concurrent and subsequent serum cholesterols of breast- and formula-fed infants. Am J Clin Nutr 28:42-45, 1975

Fungwe TV, Cagen LM, Cook GA, Wilcox HG and Heimberg M. Dietary cholesterol stimulates hepatic biosynthesis of triglyceride and reduces oxidation of fatty acids in the rat. J Lipid Red 34:933-941, 1993

Garbagnati E. Low serum cholesterol in children with fever. Acta Paediatr 82:98, 1993

Gil A, Pita ML, Martinez A, Molina JA et al. Effect of dietary nucleotides on the plasma fatty acids in at-term neonates. Hum Nutr Clin Nutr 40C:185-195, 1986

Gil A. Human milk nucleotides: influences on the lipid metabolism of newborn infants, in Symposium 582, Human milk recent knowledge. XVII International Congress of Pediatrics. Manila, 1983

Goodman DS, Smith FR, Seplowitz AH, Ramarkrishan R and Dell RB. Prediction of the parameters of whole body cholesterol metabolism in humans. J Lipid Res 21:699-713, 1980

Goodman DS, Noble RP and Dell RB. Three-pool model of the long-term turnover of plasma cholesterol in man. J Lipid Res 14:178-188, 1973

Green MH, Kohner EL and Green JB. Influence of dietary fat and cholesterol on milk lipids and on cholesterol metabolism in the rat. J Nutr 111:276-286, 1981

Grundy SM, Barrett-Connor E, Rudel LL, Miettinen T and Spector AA. Workshop on the impact of dietary cholesterol on plasma lipids and atherosclerosis. Arteriosclerosis 8:95-101, 1988

Grundy SM, Ahrens Jr EH and Davignon J. The interaction of cholesterol absorption and cholesterol synthesis in man. J Lipid Res 10:304-315, 1969

Gylling H and Miettinen TA. Cholesterol absorption and synthesis related to low density lipid metabolism during varying cholesterol intake in men with different aopE phenotypes. J Lipid Res 33:1361-1371, 1992

Hahn P, Girard J, Assan J, Kervin A and Kolodovsky O. Late effects of premature weaning to different diets in the rat. J Nutr 108:1783-1787, 1978

Hahn P, Girard J, Assan R, Frolich J and Kervran A. Control of blood cholesterol levels in suckling and weanling rats. J Nutr 107:2062-2066, 1977

Hahn P and Koldovsky O. Late effect of premature weaning on blood cholesterol levels in adult rats. Nutr Rep Int 13:87-91, 1976

Hahn P and Kirby L. Immediate and late effects of premature weaning and of feeding a high fat or high carbohydrate diet to weanling rats. J Nutr 103:609-696, 1973

Hamilton JJ, Phang M and Innis SM. Elevation of plasma lathosterol, as an indicator of increased cholesterol synthesis, in preterm (23-32 weeks gestation) in infants given intralipid. Pediatr Res 31:186-192, 1992

Hamilton JJ, Synnes A and Innis SM. Plasma cholesterol and lathosterol levels in term infants in the early neonatal period. Pediatr Res 31:396-400, 1992

Hodgson PA, Ellefson RD, Elveback LR, Harris LE, Nelson RA and Weidman WH. Comparison of serum cholesterol in children fed high, moderate, or low cholesterol milk diets during the neonatal period. Metabolism 25:739-746, 1976

Hopkins PN. Effects of dietary cholesterol on serum cholesterol: a meta-analysis and review. Am J Clin Nutr 55:1060-1070, 1992

Huttunen JK, Saarinen UM, Kostiainen E and Siimes MA. Fat composition of the infant diet does not influence subsequent serum lipid levels in man. Atherosclerosis 4786-4794, 1983

Innis SM, Quinlan P and Dierson-Schade D. Saturated fatty acid chain length and positional distribution in infant formula: effects on growth and plasma lipids and ketones in piglets. Am J Clin Nutr 57:382-390, 1993

Innis SM. Changes in plasma cholesterol density distribution of young adult male rats fed a high fat and cholesterol diet following maternal cholestyramine treatment. J Nutr 119:373-179, 1989

Innis SM. Effect of diet during pregnancy and lactation on the activity of HMG-CoA reductase in the developing rat. J Nutr 118:1177-1183, 1988

Innis SM. The role of diet during development on the regulation of adult cholesterol homeostasis. Can J Physiol Pharmacol 63:557-564, 1985

Jacobs Jr DR, Anderson JT, Hannan P, Keys A and Blackburn H. Variability in individual serum cholesterol response to change in diet. Arteriosclerosis 3:349-356, 1983

Jarvenpaa A, Raiha NCR, Rassin DK, Gaull GE. Milk protein quality in the term infant. I Metabolic responses and effect on growth. Pediatrics 70:214-220, 1982

Jensen RG. Lipids in human milk - composition and fat-soluble vitamins. In: Textbook of gastroenterology and nutrition in infancy. 2nd edition. New York: Raven Press, 157-208, 1989

Jones PJH, Lichtenstein AH and Schaefer EJ. Interaction of dietary fat type and cholesterol level on cholesterol synthesis measured using deuterium incorporation . J Lipid Res (in press) 1994

Jones PJH, Leitch CA, Li ZC and Connor WE. Human cholesterol synthesis measurement using deuterated water: theoretical and procedural considerations. Arterio and Thromb 13:247-253, 1993

Jones PJH, Leitch CA and Pederson RA. Meal-frequency effects on plasma hormone concentrations and cholesterol synthesis in humans. Am J Clin Nutr 57:868-874, 1993

Jones PJH, Main BF and Frolich JJ. Response of cholesterol synthesis to cholesterol feeding in men with different apolipid E genotypes. Metabolism 42:1065-1071, 1993

Jones PJH, Pappu AS, Illingworth DR and Leitch CA. Correspondence between plasma mevalonic acid levels and deuterium uptake in measuring human cholesterol synthesis. Eur J Clin Invest 22:609-613, 1992

Jones PJH and Leatherdale ST. Stable isotopes in clinical research: safety reaffirmed. Clinical Science 80:277-280, 1991

Jones PJH, Hrboticky N, Hahn P and Innis SM. Comparison of breast-feeding and formula-feeding on intestinal and hepatic cholesterol metabolism in neonatal pigs. Am J Clin Nutr 51:979-984, 1990

Jones PJH. Stable isotopes in nutrition research: historical perspective and overview. Can J Physiol Pharmacol 68:935-937, 1990

Jones PJH. Use of deuterated water for measurement of short-term cholesterol synthesis in humans. Can J Physiol Pharmacol 68:955-959, 1990

Jones PJH, Ridgen JE and Benson AP. Influence of dietary fatty acid composition on cholesterol synthesis and esterification in hamsters. Lipids 25:815-820, 1990

Jones PJH, Scanu AM and Schoeller DA. Plasma cholesterol synthesis using deuterated water in humans: effect of short-term food restriction. J Lab Clin Med 8:627-633, 1988

Kallio MJT, Siimes MA, Perheentupa J, Salmenpera L and Miettinen TA. Cholesterol and its precursors in human milk during prolonged exclusive breast-feeding. Am J Clin Nutr 50:782-785, 1989

Kempen HJM, de Knijff P, Boomsma DI, van der Voort KH, Gevers Leuven JA and Havekes L. Plasma levels of lathosterol and phytosterols in relation to age, sex, anthropometric parameters, plasma lipids, and apolipid E phenotype, in 160 Dutch families. Metabolism 40:604-611, 1991

Kempen HJM, Glatz JFC, Gevers Leuven JA, van der Voort HA and Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. J Lipid Res 29:1149-1155, 1988

Kiel DP, Baron JA, Plymate SR and Chute CG. Sex hormones and lipids in men. Am J Med 87:3539, 1989

Klein PD, Jiang J, Wong WW, Hachey DL and Insull W. In vivo demonstration of cholesterol synthesis inhibition by cholesterol feeding in man. Am J Clin Nutr 51(supplement):516A, 1989

Kolacek S, Kapetanovic T, Zimolo A and Luzan. Infant feeding as an important determinant of cardiovascular risk factors in adults: and 18 year follow-up study. J Pediatr Gastroenterol Nutr 13:317A, 1991

Kris-Etherton PM, Layman DK, Tork PV and Frantz ID. The influence of early nutrition on the serum cholesterol of the adult rat. J Nutr 109:1244-1257, 1979

Kubota A. Nutritional study of nucleotide components in the milk. Acta Paed Jap73:197-205, 1969

Kummerow FA, Kim Y, Hull, Pollard J, Ilinov P, Dorossiev DL and Valek J. The influence of egg consumption on the serum cholesterol level in human subjects. Am J Clin Nutr 30:664-673, 1977

Kurowska EM and Carroll KK. Essential amino acids in relation to hypercholesterolemia induced in rabbits by dietary casein. J Nutr 120:831-836, 1990

Lammi-Keefe CJ and Jensen RG. Lipids in human milk: A review. 2: Composition and fat-soluble vitamins. Pediatr Gastroenterol Nutr 3:172-198, 1984

Leitch CA and Jones PJH. Measurement of triglyceride synthesis in humans using deuterium oxide and isotope ratio mass spectrometry. Biol Mass Spectrom 20:392-396, 1991

Li JR, Bale K and Kottke BA. Effect of neonatal modulation of cholesterol homeostasis on subsequent response to cholesterol challenge in adult guinea pig. J Clin Invest 65:1060-1068, 1980

Li JR, Bale LK and Subbiah MTR. Effect of enhancement of cholesterol degradation during neonatal life of guinea pig on its subsequent response to dietary cholesterol. Atherosclerosis 32:93-98, 1979

Lin DS and Connor WE. The long-term effects of dietary cholesterol upon the plasma lipids, lipids, cholesterol absorption, and the sterol balance in man: the demonstration of feedback inhibition of cholesterol biosynthesis and increased bile acid excretion. J Lipid Res 21:1042-1052, 1980

Liu GCK, Ahrens Jr EH, Schreibman PH, Samuel P, McNamara DJ, Crouse JR. Measurement of cholesterol synthesis in man by isotope kinetics of squalene. Proc Natl Acad Sci (USA) 72:4612-4616, 1975

Lofland HB, Clarkson TB, St Clair RW and Lehner NDM. Studies on the regulation of plasma cholesterol levels in squirrel monkeys of two genotypes. J Lipid Res 13:39-47, 1972

London IM and Rittenberg D. Deuterium studies in normal man. J Biol Chem 184:687-691, 1950

Marmot MG, Page CM, Atkin SE and Douglas JWB. Effect of breast-feeding on plasma cholesterol and weight in young adults. J Epidemiol Community Health 34:164-167, 1980

Mattson FH, Benedict JH, Martin JB, Beck LW. Intermediates formed during the digestion of triglycerides. J Nutr 48:335, 1952

McNamara DJ, Kolb R, Parker TS, Batwin H, Samuel P, Brown CD and Ahrens Jr EH. Heterogeneity of cholesterol homeostasis in man. Response to changes in dietary fat quality and cholesterol quantity. J Clin Invest 79:1729-1739, 1987

Meijer GW, Van der Palen JGP, De Vries H, Kempen HJM, Van der Voort HA, Van Zutphen LFM and Beynen AC. Evaluation of the use of serum lathosterol concentration to assess whole-body cholesterol synthesis in rabbits. J Lipid Res 33:281-286, 1992

Meinertz H, Nilausen K and Faergeman O. Effects of dietary proteins on plasma lipid levels in normal subjects : interaction with dietary cholesterol. J Nutr Sci Vitaminol 36(Suppl)5157-5164, 1990

Mellies MJ, Ishikawa TT, Gartside P, Burton K, MacGee J, Allen K, Steiner PM and Brady D. Effects of varying maternal dietary cholesterol and phytosterol in lactating women and their infants. Am J Clin Nutr 31:1347-1354, 1978

Miettinen TA, Tilvis RS, and Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J of Epidemiology 131:20-30, 1990

Miettinen TA and Kesaniemi YA. Cholesterol absorption: regulation of cholesterol synthesis and elimination and within-population variations of serum cholesterol levels. Am J Clin Nutr 49:629-635, 1989

Miettinen TA. Diurnal variation of cholesterol precursors squalene and methyl sterol in human plasma lipids. J Lipid Res 23:466-473, 1982

Mott GE, Jackson EM and McMahan CA. Effects of dietary cholesterol, type of fat, and sex on bile lipid composition of adult baboons. Am J Clin Nutr 56:511-516, 1992

Mott GE, Jackson EM, McMahan CA and McGill HC. Cholesterol metabolism in adult baboons is influenced by infant diet. J Nutr 120:243-251, 1990

Mott GE, McMahan CA, Kelley JL, Farley CM, McGill HC Jr. Influence of infant and juvenile diets on serum cholesterol, lipid cholesterol, and apolipid cholesterol concentrations in juvenile baboons. Atherosclerosis 45:191-202, 1982

Naseem SM, Khan MA, Heald FP and Nair PP. The influence of cholesterol and fat in maternal diet of rats on the development of hepatic cholesterol metabolism in the offspring. Atherosclerosis 36:1-8, 1980

Nestel PJ and Kudchodkar B. Plasma squalene as an index of cholesterol synthesis. Clin Sci Mol Med 49:621-624, 1975

Nestel PJ and Poyser A. Changes in cholesterol synthesis and excretion when cholesterol intake is increased. Metabolism 25:1591-1599, 1976

Oh SY and Miller LT. Effect of dietary egg on variability of plasma cholesterol levels and lipid cholesterol. Am J Clin Nutr 42:421-431, 1985

Ohta T, Ikeda Y, Nakamura R and Matsuda I. Lipid-containing apolipid A-I: sex-related quantitative and qualitative changes in this lipid subspecies after ingestion of fat. Am J Clin Nutr 56:404-409, 1992

Overturf ML, Smith SA, Gotto AM, Morrisett JD, Tewson T, Porrman J and Loose-Mitchell DS. Dietary cholesterol absorption and sterol and bile acid excretion in hypercholesterolemia-resistant white rabbits. J Lipid Res 31:2019-2027, 1990

Parker TS, McNamara DJ, Brown CD, Kolb R, Ahrens Jr EH, Alberts AW, Tobert J, Chen J and DeSchepper PJ. Plasma mevalonate as a measure of cholesterol synthesis in man. J Clin Invest 74:795-804, 1984

Parodi PW. Positional distribution of fatty acids in triglycerides from milk of several species of mammals. Lipids 17:437-442, 1982

Paul R, Ramesha CS and Ganguly J. On the mechanism of hypocholesterolemic effects of polyunsaturated lipids. Adv Lipid Res 17:155-171, 1980

Peng S, Ho K and Taylor CB. Biologic effects of prolonged exposure to deuterium oxide. Arch Path 94:81-89, 1972

Poledne R, Scheithauer E, Bicanova H. Accelerated cholesterol synthesis in the monocytes of young myocardial infarction survivors. Cor Et Vasa 35(3):99-101, 1993

Porter MW, Yamanaka W, Carlson S, Flynn M. Effects of dietary egg on serum cholesterol and triglycerides of human males. Am J Clin Nutr 30:490-495, 1977

Potter JM and Nestel PJ. The effects of dietary fatty acids and cholesterol on the milk lipids of lactating women and the plasma cholesterol of breast-fed infants. Am J Clin Nutr 29:54-60, 1976

Quarfordt SH. Methods for the in vivo estimation of human cholesterol dynamics. Am J Clin Nutr 30:967-974, 1977

Quintao ECR and Sperotto G. The role of dietary cholesterol in the regulation of human body cholesterol metabolism. Adv Lipid Res 22:173-188, 1981

Quintao E, Grundy SM and Ahrens EH. Effects of dietary cholesterol on the regulation of total body cholesterol in man. J Lipid Res 12:233-247, 1971

Reiser R, O'Brien BC, Henderson GR and Moore RW. Studies on a possible function for cholesterol in milk. Nutr Rep Int 19:835-849, 1979

Reiser R, Henderson GR and O'Brien BC. Persistence of dietary suppression of 3-hydroxy-3-methylglutaryl coenzyme-A reductase during development in rats. J Nutr 107:1131-1138, 1977

Reiser R and Sidelman Z. Control of serum cholesterol homeostasis by cholesterol in the milk of the suckling rat. J Nutr 102:1009-1016, 1972

Reiser R. Control of adult serum cholesterol by the nutrition of the suckling: a progress report. Circulation 44 (Supplement 2):3, 1971

Riedel M, Rafflenbeul W and Lichtlen P. Ovarian sex steroids and atherosclerosis. Clin Invest 71:406-412, 1993

Rittenburg D, Schoenheimer R. Deuterium as an indicator in the study of intermediary metabolism. XI Further studies on the biological uptake of deuterium into organic substances with special reference to fat and cholesterol formation. J Biol Chem 121:235-253, 1937

Roberts DCK and West CE. Influence of rabbit milk upon cholesterolemic response of offspring of rabbits. Lipids 9:495-497, 1974

Roberts SL, McMurry MP, Connor WE.Does egg feeding (i.e. dietary cholesterol) affect plasma cholesterol levels in humans? The results of a double-blind study. Am J Clin Nutr. 34:2092-2094, 1981

Robins SJ, Fasulo JM, Lessard PD and Patton FM. Hepatic cholesterol synthesis and the secretion of newly synthesized cholesterol in bile. Biochem J 289:41-44, 1993

Roe RP, Jones PJH, Frohlich JJ and Schoeller DA. Association between apolipid E phenotype and endogenous cholesterol synthesis as measured by deuterium uptake. Cardiovas Res 25:249-255, 1991

Safonova IG, Sviridov DD, Roytman A, Rytikov FM, Dolgov VV, Nano JL, Rampal P and Repin VS. Cholesterol uptake in the human intestine. Hypo- and hyperresponsiveness. Biochimica et biophysica Acta 1166:313-316, 1993

Samman S, Kurowska EM, Khosla P and Carroll KK. Effects of dietary protein on composition and metabolism of plasma lipids in rabbits. J Nutr Sci Vitaminol 36 (Suppl) 595-599, 1990

Sanchez-Pozo A, Pita ML, Martinez A, Molina JA, Sanchez-Medina F and Gil A. Effects of dietary nucleotides upon lipid patterns of newborn infants. Nutr Res 6:763, 1986

Savaiano DA and Clifford AJ. Adenine, the precursor of nucleic acids in intestinal cells unable to synthesize purines de novo. J Nutr 111:1816-1822, 1981

Schoeller DA, Peterson DW and Hayes J. Double-comparison method for mass spectrometric determination of hydrogen isotopic abundances. Anal Chem 55:827-832, 1983

Schonfeld G, Patsch W, Rudel LL, Nelson C, Epstein M and Olson RE. Effects of dietary cholesterol and fatty acids on plasma lipids. J Clin Invest 69:1072-1080, 1982

Schwartz CC, Zech LA, VandenBroek JM and Cooper PS. Cholesterol kinetics in subjects with bile fistula. J Clin Invest 91:923-938, 1993

Slater G, Mead J, Dhopeshwarker G, Robinson S, Alfin-Slater RB. Plasma cholesterol and triglyceride in men with added eggs in the diet. Nutr Rept Int 14:249-260, 1976

Small DM. The effect of glyceride structure on absorption and metabolism. Annu Rev Nutr 11:413-434, 1991

Sodhi HS, Kudchodkar BJ and Mason DJ. Cholesterol synthesis. In Monographs on Atherosclerosis Vol 9: Clinical methods in the study of cholesterol metabolism. Kritchevsky D and Pollack OJ, editors. S Karger Publishers, New York, N.Y. 57-86, 1979

Spear ML,. Hamosh M, Bitman J, Spear ML and Wood DL. Milk and blood fatty acid composition during two lactations in the same woman. Am J Clin Nutr 56:65-70, 1992

Taylor CB, Mikkelson B, Anderson JA and Forman DT. Human serum cholesterol synthesis measured with the deuterium label. Arch Pat 81:213-231, 1966

Tomarelli RM, Meyer BJ, Weaber JR and Bernhart FW. Effect of positional distribution on the absorption of the fatty acids of human milk and infant formulas. J Nutr 95:583-590, 1968

Waddell CA and Desai ID. The use of laboratory animals in nutrition research. Wld Rev Nutr Diet 36:206-222, 1981

Whatley BJ, Green JB and Green MH. Effect of dietary fat and cholesterol on milk composition, milk intake and cholesterol metabolism in the rabbit. J Nutr 111:432-441, 1981

Wilkinson, Leland SYSTAT: The system for statistics. Evanston IL: SYSTAT, Inc., 1990

Wong WW, Hachey DL, Insull W, Opekun AR, Klein PD. Effect of dietary cholesterol on cholesterol synthesis in breast-fed and formula-fed infants. J Lipid Res 34:1403-1411, 1993

Wong WW, Hachey DL, Feste A, Leggitt J, Clarke LL, Wilson GP and Klein PD. Measurement of in vivo cholesterol synthesis from 2H20: a rapid procedure for the isolation, combustion, and isotopic assay of erythrocyte cholesterol. J Lipid Res 32:1049-1956, 1991

Woodward DR, Rees B and Boon JA. Human milk fat content: within-feed variation. Early Hum Dev 19:39-46, 1989

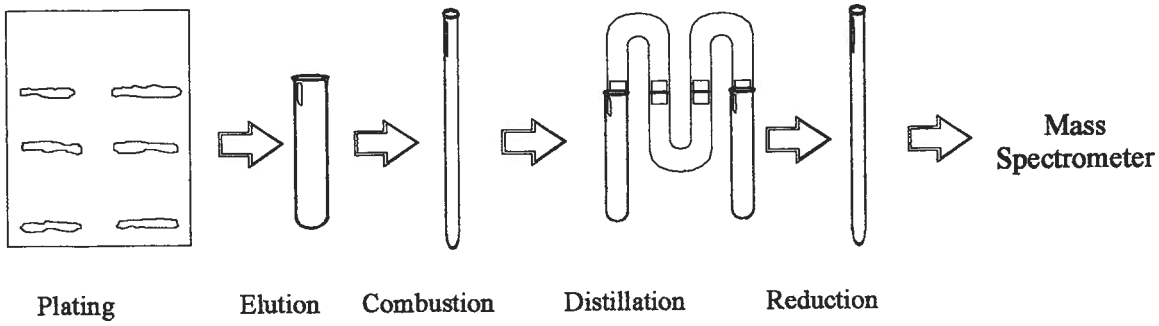
Zar JH. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J. 1984. pp 126-128, 132-136, 168-173.

Appendix 1 - Pilot Study

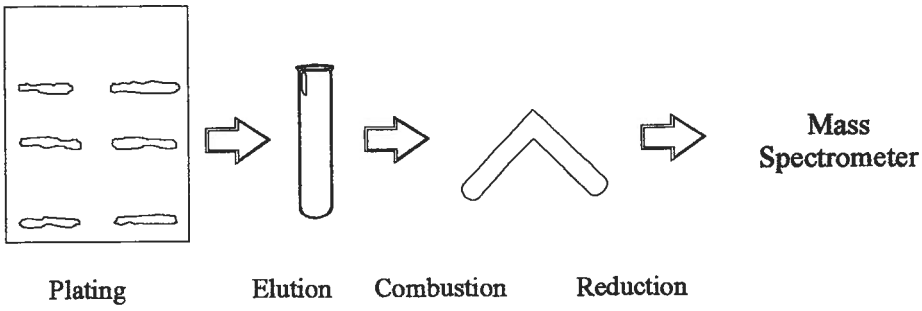
An attempt to modify the methodology in order to cut down on labor and avoid steps which may introduce error was carried out by combining the combustion and distillation procedures into one step. Procedures are outlined in Appendix 2. After removal of the solvent from the tube containing the cholesterol, the tube was removed from the vacuum apparatus, heated in the center with a hydrogen torch, and bent to approximately a 90° angle. The zinc (60 mg) was added near the open end of the tube, reapplied to the vacuum apparatus, and allowed to return to baseline. The tube was then sealed with the hydrogen torch, and removed while keeping the copper at one end of the tube and the zinc at the other. The copper end was then placed into a 540°C oven for four hours to combust the cholesterol, while keeping the zinc end outside the oven door. An attempt was then made to freeze out the water at the end of the tube containing the zinc, while leaving the $C0_2$ behind at the end with the copper. The tube was then sealed with a hydrogen torch at the bend, leaving the zinc end of the tube, which was reduced in a 540°C oven for 30 minutes. This eliminated the fairly lengthy distillation procedure. Mass spectrometry analysis of these samples, however, produced very erratic results. Cross-contamination between the zinc and copper or between the $C0_2$ and water may have been the factors responsible.

Appendix 2: Schematic representation of conventional system and pilot system

Conventional System:



Pilot System:



Appendix 3 - Blood Sample Sizes

Purified cholesterol was dissolved in a set quantity of chloroform. From this, specific amounts were pipetted into combustion tubes, to result in graduated quantities for analysis (0.75, 1.0, 1.5, 2.0, and 2.5 mg). Chloroform was removed under vacuum, the tube sealed , combusted and distilled, as described earlier for cholesterol samples from blood. Pressure measurements were taken from the mass spectrometer along with deuterium enrichment, and a calibration curve drawn.

Appendix 4 - Fatty Acid Determination of Milk

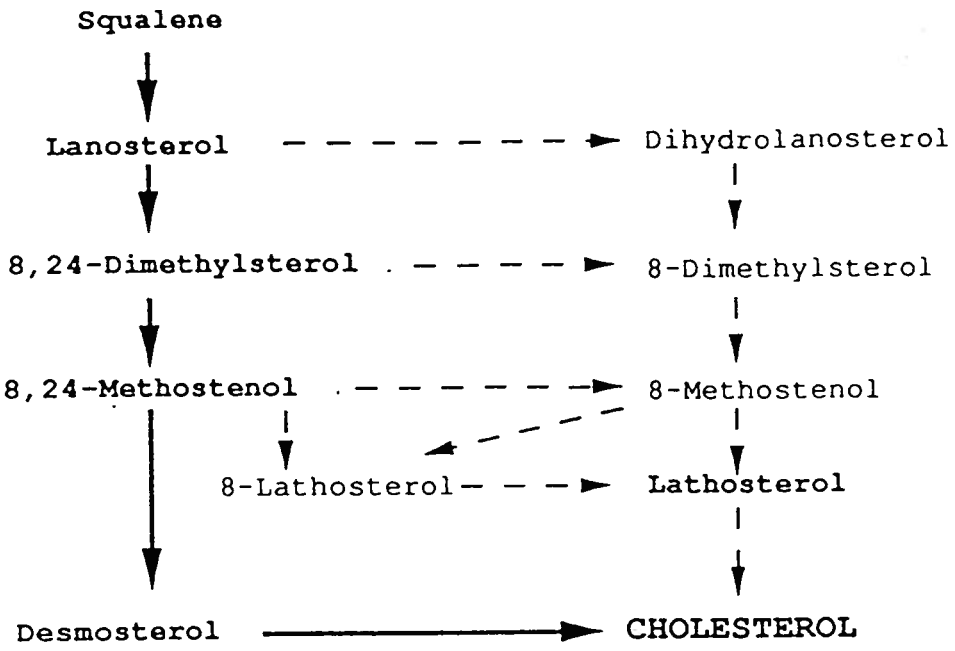
1. Lipid Extraction:

A sample (200-500 uL) of each of the two formulae and two breast milk samples (obtained from two different mothers of infants involved in the study in Cincinnati) was combined with 15 mL chloroform/methanol + BHT (2:1 v/v). C17 standard (500 uL) was added and the mixture shaken for 10 minutes and then centrifuged for 10 minutes at 1500 g, upon which the upper phase was removed. Methanol/chloroform (2:1 v/v) (15 mL) was added to the original mixture, shaken for 10 minutes, and centrifuged for 10 minutes. The upper phase was again removed and combined with the first. Potassium chloride in distilled water (7.5 mL 0.88%) was added to the combined extracts. The mixture was shaken for 15 minutes and then centrifuged for 10 minutes. The upper layer was aspirated off, leaving the lower phase which contained the purified lipid sample. The solvent was evaporated with nitrogen, leaving the lipid fraction.

2. Methylation for Gas Liquid Chromatography (GLC):

KOH in methanol + BHT (2 mL 0.5 M) was added to each sample and boiled at 100°C for 20 minutes. Cooling for 5 minutes followed, upon which 2 mL of 12% Boron-Trifluoride in methanol (Kodak Canada) was added. Boiling was continued for an additional 25 minutes. Again, cooling for 5 minutes was allowed, upon which 2 mL of hexane and 4 mL of distilled water saturated with potassium chloride were added. The mixtures were shaken for 10 minutes, centrifuged for 10 minutes and the top organic phase removed. Two mL of hexane was again added to the sample, the mixture shaken for 10 minutes and centrifuged for 10 minutes. The upper organic layer was removed and added to the first extraction. A portion of the combined extract was transferred to a small vial and sealed for GLC analysis.

Appendix 5 - Cholesterol synthesis in the human mammary gland



Kallio et al., 1989

APPENDIX 6 - Sample calculation for correction of deuterium enrichment values for small blood samples

	Pressure	D_2O/H_2O
1 mL RBCs	0.811×10^{-9}	-53.378
2 mg cholesterol standard	1.540×10^{-9}	-158.165

$y = -185.351 + 41.867(1/x)$

a) $y = -185.351 + 41.867(1/0.811) = -133.727$

b) $y = -185.351 + 41.867(1/1.540) = -158.165$

$b/a = -158.165/-133.727 = 1.18$

The original deuterium enrichment value for the blood sample (-153.378×10^{-9}) is now multiplied by a factor of 1.18 to give a new corrected value of -63.132.

Appendix 7 - Correction for small blood samples

Deuterium Enrichment	Correction Factor	Recalculated Deuterium Enrichment
-161.514	1.24	-200.277
-166.219	1.11	-184.503
-122.956	1.17	-143.859
-126.065	1.17	-147.496
-108.397	1.21	-131.160
-112.358	1.22	-137.077
-171.159	1.08	-184.852
-169.693	1.08	-183.268
-40.661	1.31	-53.266
-36.450	1.21	-44.105
44.557	1.32	58.815
48.222	1.48	71.369
-202.532	1.23	-249.114
-83.725	1.19	-99.633
-81.435	1.25	-101.794
46.250	1.20	-55.500
-116.308	1.22	-141.896
-48.091	1.18	-56.747
-58.664	1.18	-69.224
-33.009	1.20	-39.611
-175.331	1.03	-180.591
-201.924	1.05	-212.020
-77.534	1.19	-92.265
-90.132	1.14	-102.750
-22.634	1.27	-28.745
-30.645	1.16	-35.548

APPENDIX 8 - Fatty acid composition* of infant formula (regular and modified) and human milk

Fatty Acid	Mature Human Milk		% Formula Regular		Formula Modified	
C6:0	0.1 ± 0.1	---	---	---	---	---
C8:0	0.2 ± 0.1	---	1.9	---	1.9	---
C10:0	1.3 ± 0.2	---	1.6	---	1.6	---
C12:0	4.3 ± 0.4	---	11.9	---	11.9	---
C14:0	7.8 ± 2.0	(4.3 ± 0.4)	5.9	(6.4 ±0.5)	5.9	(6.8 ± 0.3)
C16:0	22.0 ± 2.0	(21.6 ± 0.7)	13.8	(15.4 ± 0.2)	13.8	(16.3 ± 0.3)
C16:1	2.5 ± 0.3	(1.6 ± 1.8)	1.5	(0.5 ± 0.7)	1.5	(1.3 ± 0)
C18:0	7.2 ± 0.3	(6.5 ± 1.0)	7.9	(8.7 ± 0.2)	7.9	(8.8 ± 0.1)
C18:1	35.0 ± 2.0	(36.6 ± 0.7)	35.5	(44.0 ± 0.2)	35.5	(40.1 ± 0.5)
C18:2 (w6)	10.0 ± 2.0	(17.6 ± 2.1)	16.2	(14.7 ± 0.3)	16.2	(17.3 ± 0.5)
C18:3 (w3)	1.1 ± 0.2	---	2.3	(1.6 ± 0.3)	2.3	(2.0 ± 0.2)
C20:0	1.0 ± 0.1	---	0.5	---	0.5	---
C20:4 (w6)	1.0 ± 0.1	---	0.18	---	0.18	---
P:S ratio	0.32:1.0		0.33:1		0.33:1	
Cholesterol mg/dL	12.0		3.3		13.3	

Mean ± standard deviation
( ) = analysis done at the University of British Columbia
* Wyeth-Ayerst Laboratories, Philadelphia, USA

Appendix 9 - Nutrient composition of infant formula used

Nutrient	Units	Label Claim (per 100 ml)	Initial Results1 (per 100 ml)
Proximates:			
Fat	g	3.6	3.5
Protein	g	1.5	1.5
Carbohydrate	g	7.2	7.3
Ash	g	0.25	0.29
Vitamins and Nucleotides:			
Vitamin A	IU	200	306
β-Carotene	IU	21.2*	20.5
Vitamin D	IU	40	49
Vitamin E	IU	0.95	1.33
Vitamin K1	µg	5.5	9
Thiamine	µg	67	106
Riboflavin	µg	100	194
Vitamin B6	µg	42	43
Vitamin B12	µg	0.13	0.23
Vitamin C	mg	5.5	11.1
Niacinamide	µg	500	522
d-Pantothenic Acid	µg	210	369
Folic Acid	µg	5	6.8(2)
Biotin	µg	1.5	3.7
Choline	mg	10	14
Taurine	mg	3.76	5.64
Cholesterol	mg	—	—
			13.0**
Cytidine-5'-Monophosphate	mg	2.47*	2.74
Uridine-5'-Monophosphate	mg	0.75*	0.7
Guanosine-5'-Monophosphate	mg	0.29*	0.26
Inosine-5'-Monophosphate	mg	0.28*	0.25
Adenosine-5'-Monophosphate	mg	0.76*	0.79
Minerals:			
Calcium	mg	42	45
Phosphorus	mg	28	33
Iron	mg	1.2	1.3
Magnesium	mg	4.5	6.2
Potassium	mg	56	69
Sodium	mg	15	16
Chloride	mg	37.5	39.4
Iodine	µg	6	9
Copper	µg	47	47
Zinc	mg	0.5	0.6
Manganese	µg	10	27

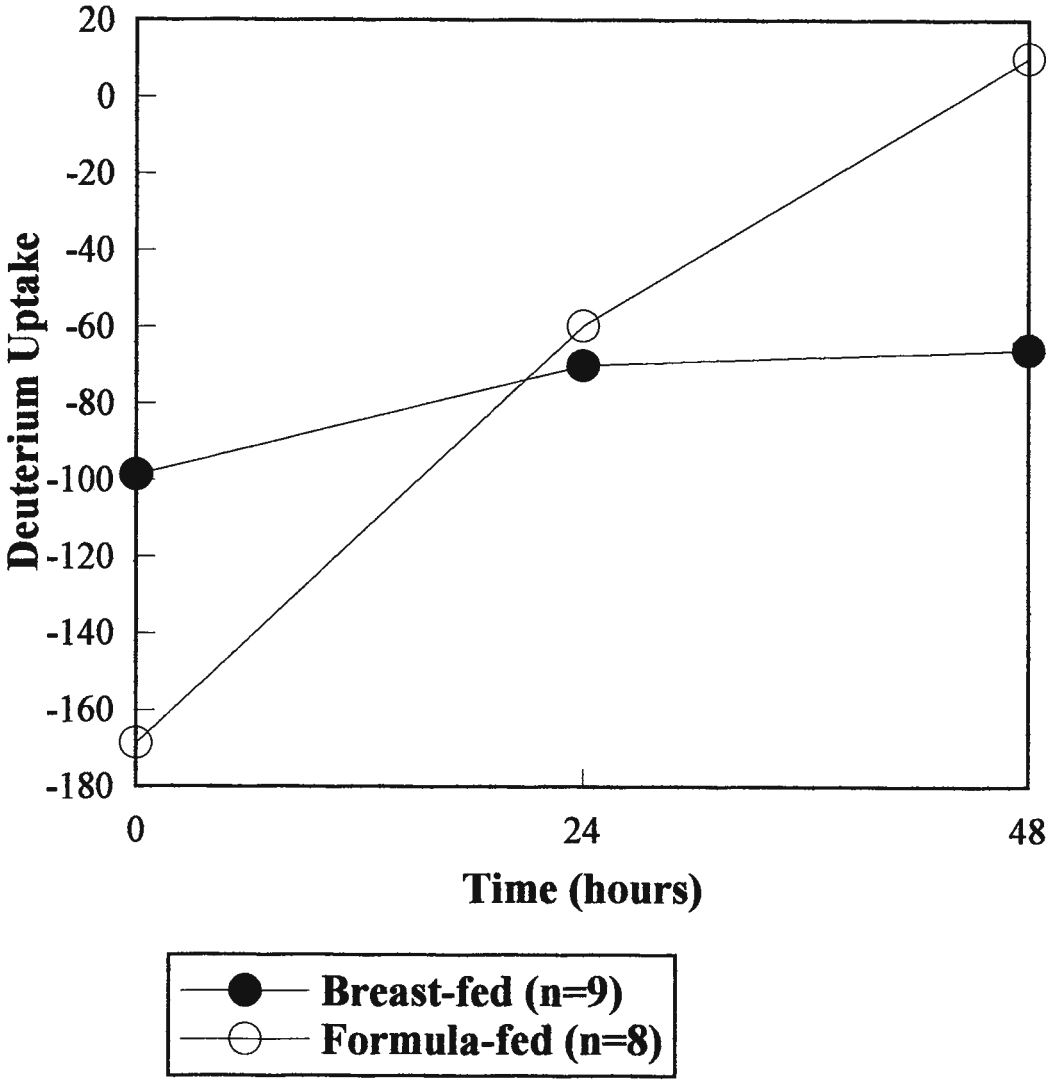
* Intended level, not a label claim

** Modified infant formula

(1) Data generated by Wyeth-Ayerst Canada (Windsor) unless otherwise indicated

(2) Data generated by Hazleton Laboratory, Wisconsin

Appendix 10 - Deuterium uptake of breast-fed and formula-fed infants at 4 months



Appendix 11 - Deuterium enrichment of breast-fed and formula-fed infants at 4 months

	Hours Post Loading					
	0	0	24	24	48	48
	Breast	Formula	Breast	Formula	Breast	Formula
	-48.096	-170.426	-11.002	-38.556	-52.858	46.39
	-79.779	-136.186	-50.465	-18.379	-56.204	22.801
	-53.837	-103.086	-60.909	-83.994	-38.839	-21.124
	-132.163	-163.653	-118.501	-54.146	-105.815	46.25
	-132.894	-202.532	-67.557	-82.58	-31.065	-26.64
	-76.577	-188.628	-54.207	-83.833	-110.378	-41.02
	-112.006	-193.262	-89.126	-97.012	----	44.845
	-87.694	-191.056	-53.378	-18.999	----	----
	-163.867	----	-124.511	----	----	----
Mean	-98.546	-168.604	-69.962	-59.687	-65.860	10.215
Standard Deviation	39.139	33.881	35.648	31.465	34.004	38.579

APPENDIX 12 - Study instruction form

CHOLESTEROL STUDY

Dear Parents:

Congratulations on your new baby! We are glad that your son is healthy and able to go home with you soon.

The health of all children is our major concern and infant nutrition plays an important role in their well-being. Especially today, there is a lot of concern about cholesterol and how it affects health. We would like to use this opportunity to tell you about a new study on infants and how their diet early in life may affect how they handle cholesterol when they grow up.

There are many kinds of milk available, each with different amounts of cholesterol in them: Human breast milk has the highest, soy milk formula has the lowest and cow's milk formula in between. Human milk has between 5 - 10 times more cholesterol than cows milk formulas. We recently finished a study at Children's Hospital where we showed that breast fed infants at 4 months of age are making less cholesterol than formula fed infans. They are taking more cholesterol but making less. We want to know if babies who receive more cholesterol early in life will continue to make less cholesterol later in life. This might result in lower blood cholesterol and decrease the risk of heart diseases. If this is true, then we might have to add more cholesterol to infant formulas. This study will find that out.

We are glad to know that you have chosen to bottle feed your son. If you agree to this study, we will follow him for either four months or for one year. At the end of the study we will take blood (three times, about 1 1/2 teaspoonfuls each time) and give him a special kind of water called "deuterated water" or "heavy water" that will help us measure how little or much cholesterol he makes in his body. This water is harmless to your baby. This test will be done both at 11 and 12 months if the baby is in the study for the whole year. You also have to keep a diet diary, for three days every month while your baby is in the study. We will send you forms to fill out.

We provide all the formula free while your baby is in the study. Half of the formula-fed babies will be in regular SMA formula, the other half on SMA which has the same amount of cholesterol as breast milk. If your baby is in the study for a whole year, we will also provide coupons for baby food. You will also have to give him extra cholesterol between 11 and 12 months, the same amount as in one egg. This study has been approved by the Hospital's Institutional Review Board.

We hope that will have sparked your interest in this exciting study. If you want more information about the study or if you decide to participate in the study, please call us at 558-4903. Thank you!

Sincerely,

Susan Krug-Wispé, R.D.
Thor Thorkelsson, M.D.