

GROWTH, THIAMIN STATUS, ERYTHROCYTE FATTY  
ACID COMPOSITION, AND VISUAL ACUITY IN  
FULLTERM INFANTS FED BREASTMILK, FORMULA,  
OR EVAPORATED MILK

CENTRE FOR NEWFOUNDLAND STUDIES

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URSULA McCLOY









**Growth, Thiamin Status, Erythrocyte Fatty Acid Composition,  
and Visual Acuity in Fullterm Infants  
Fed Breastmilk, Formula, or Evaporated Milk**

A Thesis presented to  
the Department of Biochemistry  
of  
Memorial University of Newfoundland

by

Ursula McCloy

In partial fulfillment of the  
requirements for the degree of  
Masters of Science

May, 1996

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ISBN 0-612-13925-5

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## ABSTRACT

The effects of feeding breastmilk (BM), commercial formulas (F), or evaporated milk formula (EM) on growth, thiamin status, erythrocyte phosphatidylethanolamine (PE) fatty acid composition and visual acuity in 100 healthy full term infants were determined. Growth, thiamin status, and erythrocyte PE fatty acid composition was determined at birth, 3, and 6 months of age. Thiamin status was assessed by determining the erythrocyte transketolase activity (TKA) and the fatty acid composition of milk and blood was determined by gas-liquid-chromatography. Preferential looking acuity was assessed at 3 and 6 months of age using the acuity card procedure.

There were no significant differences in weight, length, or head circumference between any feeding groups. However, daily weight gain between three and six months was significantly lower for the BM group (weight gain:  $\bar{x} \pm \text{SD}$ , BM =  $15.5 \pm 4.0$  g, F =  $20.4 \pm 5.8$  g, EM =  $20.2 \pm 6.8$  g,  $p < 0.05$ ). As well, breastfed infants had slightly lower head circumference growth velocity between three and six months of age.

There were no differences in the thiamin pyrophosphate effect between groups, however, BM infants had significantly lower transketolase activity than the F group which correlated with energy but not thiamin intake.

The breastmilk obtained from mothers in the study contained on average (% total fatty acids by weight), 12.1 % linoleic acid (18:2(n-6)), 2.1% linolenic (18:3(n-3)), and 0.2% docosahexaenoic acid (22:6(n-3)). Infants in the F group consumed either Similac or Enfalac. Similac contained 30.5 % 18:2(n-6), and 4.9 % 18:3(n-3). Enfalac contained

17.1 % 18:2(n-6) and 1.8% 18:3(n-3). EM contained (% total fatty acids) 2.1% 18:2(n-6), and 0.8% 18:3(n-3). Only breastmilk contained 22:6(n-3).

In the circulation, F fed and EM fed infants had lower arachidonic acid (20:4(n-6)) at 3 months ( $p < 0.05$ ) than BM fed infants ( $\bar{x} \pm SD$ , 20.3 $\pm$ 2.8, BM, 18.3  $\pm$  2.7, F, 18.7  $\pm$  2.7, EM, % total fatty acids) but not at 6 months, and EM fed infants had lower adrenic acid (22:4(n-6)) at both 3 and 6 months. 22:6(n-3) was the highest in the BM group at both 3 (6.0  $\pm$  1.7, BM, 3.1  $\pm$  0.8, F, 4.1 $\pm$ 0.9, EM, % total fatty acids) and 6 months of age (5.3  $\pm$  1.6, BM, 2.9  $\pm$  0.8, F, and 4.2  $\pm$  1.3, EM, % total fatty acids) followed by EM then F. Visual acuity was higher in the BM group than EM (3.86  $\pm$  0.29 cycles/degree vs 3.29  $\pm$  0.41, 3 mos, 9.03  $\pm$  0.29 vs 7.54  $\pm$  0.25, 6 mos,  $p < 0.05$ ) with intermediate values in the F group (NS compared to EM and BM). Differences seen in visual acuity may be due to the low 18:3(n-3) in EM of 0.3 % of energy and are not reflective of 22:6(n-3) in circulation.

From the results of the present study it appears that EM formulas may not meet the essential fatty acid requirements for optimal visual acuity, however, it may be adequate for thiamin and optimum growth. The differences seen in the BM group in growth and TKA can be related to lower energy intakes and do not indicate any deficiencies.

## ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. James K. Frie', for his continued help, guidance, and encouragement throughout this research project. I would also like to thank Dr. G. Herzberg and Dr. W. Andrews who were not only members of the graduate supervisory committee, but also were actively engaged in the project. Dr G. Herzberg and his research assistant Craig Skinner must also be acknowledged for their allowance and assistance in conducting the fatty acid analyses in their laboratory. A special thank you as well for the cooperation of Dr. M. Courage of the Department of Psychology and her research assistant Natalie Finlay for their performance of visual acuity testing.

A very special thank you goes towards Allison McDonald and Claude Mercer who were integral to this research project.

Technical assistance from Sonia Nolan and Sean Fryer is also greatly appreciated.

I also acknowledge the laboratory technologists at the Grace General Hospital, the Janeway Child Health Centre, and the Carbonear General Hospital as well as the hospitals themselves for their cooperation and clinic space.

Most importantly, I would like to thank the parents and their infants who participated in this study, for without them the study would not have been possible.

This research was funded by Health Canada (National Health Research and Development Program, NHRDP).

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## **CHAPTER 1.0      INTRODUCTION**

### **1.1                  History of infant feeding**

Successful lactation has been a requirement for survival among all mammals. However, recently, humans have been an exception. While alternate feeding has occurred on a large scale in the past 60 years with apparent success, this is an extremely short time when compared with the overall history of human lactation. As such, it should be recognized as a relatively short term biological experiment, with unknown long term consequences (Neville et al, 1983).

Although it is assumed that alternate feeding to breastmilk is a recent development, feeding bottles have been found in excavations as far back as 2000 B.C. in France, and in the Nile Basin dated around 500 B.C. A perforated cow's horn was first mentioned in the third century in the Talmud and became common in the Middle ages in the peasant classes. In 1565, the feeding of goat's or cow's milk through a horn after the third month of life was recommended (Wickes, 1953).

The only alternatives to breastmilk before the nineteenth century were either animal milk or a form of starchy gruel. The first dehydrated milks were made in 1855, with a patent for a process of slowly concentrating sweetened cow's milk. Nestle first produced condensed milk in tin bottles in 1866, followed by several other companies. By 1883 there were 27 brands of patent infant food (Wickes, 1953).

With this increase in artificial infant food, the mortality rates also increased. The mortality rate in Paris in 1777 was 80% for infants artificially fed, and 59-82% in Berlin

versus only 9% for those breast fed in the nineteenth century (Cone, 1976)

Although other modes of infant feeding were attempted, breastfeeding was still the most common mode well into the twentieth century accompanied by late weaning ages. In a survey of US cities between 1911-1916, 58 percent still breastfed at the age of one year (Fomon, 1974).

### 1.1.1 Advancements

In the 40 year period between 1880 and 1920, several major advances occurred which made bottle feeding both economically feasible and reasonably safe for infants. These advances were mainly (Fomon, 1974):

- 1) safer water supplies and of sanitary standards for milk storage and handling;
- 2) development of easily cleansed and sterilized bottles and nipples; and
- 3) alteration of curd tension of milk.

1) *Safety of water and milk.* With the chlorination of water and garbage disposal improvements, water safety was much improved in the late 1800's. As well, general sanitation was improved with the identification of the colon-bacillus and the determination that the organisms causing bloody diarrhea were of the dysentery group. Some controlled heating of milk was introduced in Germany and the US, but pasteurization was not introduced until much later. Bacterial contamination was therefore a problem and it was not until the early part of the twentieth century, with the advent of the kitchen icebox, that the storage of milk became feasible.

In 1920, the reduction of bacterial content by the acidification of milk was

attempted. Originally, the benefits of this process was the reduction of its buffering capacity which was thought to promote bacterial growth, decrease the flow of pancreatic juice and bile, and inhibit gastric secretion. However, its most important benefit was actually a decrease in curd tension.

In the early 1900's condensing of milk was patented with sugar added to increase keeping qualities. This was used widely in infant feeding. However, it was found to be unsatisfactory as an infant food probably due to the high caloric content. The sanitary open top can allowed clean filling so that evaporated milk could be marketed in cans. However, evaporated milk was not widely used in infant feeding until the 1920's.

2) *Feeding devices.* The feeding devices consisting of spouted pots made of pottery, pewter, or silver used until the late eighteenth century were difficult to clean and cumbersome. Glass bottles enabled much more thorough cleaning. Nipples were formerly made of a tanned heifer's teat, cork, wood, or decalcified ivory. In 1864, a patent for a rubber nipple placed at the end of a flexible feeding tube was obtained. Rubber nipples directly attached to narrow mouthed glass bottles were in use by the beginning of the twentieth century.

3) *Curd tension.* When cow's milk enters the stomach the hydrochloric acid present causes the precipitation (coagulation) of the casein and calcium, which is called the curd. The remaining watery portion contains most of the lactose and whey proteins. When coagulated, cow's milk has a very high curd tension and causes gastrointestinal disturbances. Fresh human milk, however, contains little casein and forms a soft flocculent curd. The processing of cow's milk by acidification, dilution, boiling,

modification of mineral composition, and treatment with enzymes, all decrease curd tension, making the milk more digestible for the infant. These advancements in the processing of cow's milk led the way for the wide acceptance of evaporated milk and the decline of breastfeeding.

### **1.1.2 Evaporated milk introduction**

The introduction of evaporated milk in the late 1920's was heralded by leading pediatricians for its digestibility. Marriott and Schoenthal, prominent pediatricians at the time, reported the following in a study in 1929;

... evaporated milk mixtures were uniformly well digested ... There were no cases in which it was found necessary to substitute some other form of milk for the evaporated milk because of untoward symptoms or failure to do well. The results of evaporated feedings of newborn infants appear to us to indicate that this form of milk is readily digestible and well utilized by very young infants (Marriot, et al, 1929).

Even much later, in 1966, Dr. Benjamin Spock, in his infant feeding book promotes breastfeeding as a first choice but if it is not convenient, he writes;

There is nothing mysterious about a formula. It is usually a combination of evaporated or pasteurized fresh milk, water and some added sugar to make it a little closer to breastmilk in its composition.... doctors have been able,

gradually to test out simpler formulas... which have worked well and have made infant feeding more fun for babies and mothers.....Evaporated milk has become increasingly popular because it is inexpensive, convenient to keep, safe, easily digested by most babies (Spock, 1966).

The acceptance of the medical establishment of formula feeding and the lack of breastfeeding promotion allowed breastfeeding to decline, thus affecting both the nutritional status of infants and causing probable economic hardship for families.

### **1.1.3 Prevalence of feeding practices**

The prevalence of various feeding practices differs with both regional variation and income level. Data collected during the Nutrition Canada survey of 1970-1972, reported that 24% of the 895 infants surveyed consumed evaporated milk. There was shown to be a marked relationship between income level and type of milk consumed. Of those infants fed evaporated milk, 52% were in the lowest income group, 22% in the low income group, and only 8% in the "other" group (Myeres, 1979).

There was also a regional variation, with the Atlantic provinces and Quebec having a much higher rate of bottle feeding than the Pacific region. Evaporated milk formulas were fed more frequently in the Atlantic provinces than in the rest of Canada. In Atlantic Canada, 41% of infants were fed EM at one week of age (Myeres, 1979). In the US in 1972, fewer than 5% were fed evaporated milk and about 70% were fed commercial formulas (Martinez, 1976), a drop from an estimated 80% in 1960 (Cone,



1981). By 1979, in a study done in Montreal and Toronto, 71% of infants were being breastfed at 1 month of age. The mean duration of breastfeeding was 3.5 months. The rest consumed formula with whole cow's milk added to the diets gradually after one month. Evaporated milk was not a factor. It was consumed by only 2% of infants at 12 months with no apparent usage before this time (Yeung, 1981).

In Newfoundland, the use of evaporated milk in infant feeding did not become widespread until after confederation in 1949 due, in part, to the increased flow of government money. Before Confederation, breastfeeding was common, but by 1960, almost all infant feeding was evaporated milk. With this rapid increase in the use of evaporated milk came a sharp rise in infantile scurvy in Newfoundland, with 77 cases reported in 1959. Of the 40 of those 77 who answered a survey, none had breastfed their infants, and all had fed evaporated milk. The figure for breastfeeding was considered to be as low as 10%. Part of the decline was associated with the advice by doctors to stop breastfeeding when the incidence of tuberculosis was high, thus creating in some women's minds a relationship between breastfeeding and tuberculosis. As well, breastfeeding was considered a sign of poverty held over from the depression years (Severs et al, 1961).

Although it was known by doctors that breastfeeding prevented infantile scurvy, education was considered too slow a process with segments of the population too hard to reach. Therefore it was recommended to supplement evaporated milk with vitamin C instead of promoting breastfeeding (Severs et al, 1961). In March, 1964, the Federal Food and Drug Regulations were changed to allow vitamin C to be added to evaporated

milk, and the incidence of scurvy declined (Severs, 1964). With this supplementation, evaporated milk came to be seen as a protection against scurvy, even for infants. This may have further encouraged its use in infant feeding.

While the prevalence of evaporated milk feeding declined in the U.S. and Canada, it remained common in Newfoundland. In Newfoundland in 1978, Mackey and Orr found that, in hospital, 17% of infants were breastfed, 60% received formula, and 22% received evaporated milk formula. At one week of age, 43% of the metropolitan, 47% of the urban, and 72% of the rural infants were consuming evaporated milk (Mackey and Orr, 1978).

By 1992, Matthews et al (1992), found that evaporated milk feeding, although having dropped substantially, remained common in Newfoundland. This was particularly true in infants of lower income, less educated mothers. By one month of age, 32%, 55%, and 14% were fed breastmilk, commercial formulas, and evaporated milk, respectively. At four months of age, evaporated milk feeding rose to 21% of infants and breastfeeding dropped to 23%. This breastfeeding rate is about half the national average. It is therefore evident that there are cultural and traditional factors involved, as well as economical, in Newfoundland feeding practices. With a poor economy, it is doubtful that the use of evaporated milk will decline.

## **1.2 Growth and development in infancy**

The rate of gain in both weight and length is faster during the first year of life than at any other age. As seen by growth curves, the early months show very rapid growth

followed by a gradual deceleration towards the end of the year. By 12 months the infant's weight will be tripled, and length increased by 50%. Body fat increases to 25% of body weight, with body water decreasing. Physiological and metabolic functions, such as renal function, stomach capacity, and digestive ability, stabilize. Neuromuscular, social and psychological development, also occur rapidly at this time (Beal, 1980).

Anthropometric measurements are used widely in the assessment of nutritional status. Nutritional anthropometry has been defined as:

...measurements of the variations of the physical dimensions and the gross composition of the human body at different age levels and degrees of nutrition. (Jelliffe, 1966)

These measurements are particularly important when there is a chronic imbalance between intakes of protein and energy. Anthropometric indices are drawn from either a single measurement, such as weight for age, or from a combination, such as weight and height. Reference data can be obtained from either local or international sources. Local reference data is compiled from a local elite group of healthy, well-nourished individuals, whereas international data is compiled from a cross-sectional sample, using well standardized procedures, with the majority of the sample population obtaining its full growth potential (Gibson, 1990).

The World Health Organization recommends the National Centre for Health Statistics (NCHS) reference growth data as an international standard since it meets the criteria mentioned above. The values for weight, length, and weight for length from birth to 36 months are taken from longitudinal data collected by the Fels Research Institute

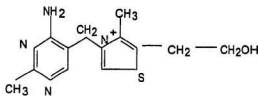
between 1960 and 1975. The measurements were taken from 720 white middle class infants and children, with the majority fed proprietary formula-based products (Gibson, 1990).

However, the appropriateness of these charts as an international standard has been questioned. Recent evidence from Britain, Canada, Australia, Finland, and the US has shown that breastfed babies match the growth charts until three months of age. However, this is followed by reduced growth velocity with breastfed infants falling below the median at 6-9 months (Whitehead et al, 1981, Chandra, 1982, Hitchcock et al, 1982, Duncan et al, 1984, Salmonpera et al, 1985, Whitehead et al, 1984, Dewey et al, 1992). The lower growth rates and energy intakes found in breastfed infants is not associated with detrimental consequences such as reduced activity level, increased morbidity, or differing behavioural development (Dewey, et al, 1991). These results suggest that NCHS growth charts may be inappropriate for breastfed infants and need to be revised.

### 1.3 Thiamin

Thiamin is a water soluble vitamin with extensive biological roles (Figure 1.1). As a component of the coenzyme thiamin pyrophosphate, it functions as a coenzyme in biochemical reactions related to carbohydrate metabolism leading to the formation of carbon dioxide (Yeung, 1983). This includes the oxidative decarboxylation of  $\alpha$ -ketoacids and pyruvate, transketolase reactions of the pentose phosphate pathway, as well as acting as a coenzyme in fatty acid synthesis. It is also involved in the decarboxylation of branched chain amino acids. Thiamin may also have a noncoenzyme role in neuronal

membrane excitability (Tsang, et al, 1988).



**Figure 1.1 Structure of Thiamin.**

### **1.3.1 Absorption and transport**

Thiamin absorption can be both active or passive, depending on the concentration. At low concentrations, it is active, occurring through a  $\text{Na}^+$ -dependent, carrier-mediated process primarily in the jejunum. At high intakes absorption is mainly passive. The rate of absorption is quite high, except in the cases of excess ethanol consumption or folate deficiency. Thiamin is converted to its active form, the phosphate ester thiamin diphosphate (TDP) (commonly known as thiamin pyrophosphate (TPP)) in the mucosal cells, and is then released into the plasma. In the brain TDP can also be converted to thiamin triphosphate (TTP) (Hunt et al, 1990).

### **1.3.2 Deficiency**

The primary result of thiamin deficiency is impaired carbohydrate metabolism, manifesting itself in beri-beri. Infantile beri-beri may occur between one and four months

of age (Tsang et al., 1988), and is characterized by diminished urinary thiamin excretion, progressive edema and acute cardiac failure, often resulting in death. Severe thiamin deficiency in developed countries is rare, with only two cases reported in Canada and the US since 1958. The cause in each of these cases was a change to unsupplemented soy milk formula, which contained almost no thiamin (Davis, et al, 1958, Cochrane, et al, 1961). Beri-beri has also been found in breastfed infants of thiamin deficient mothers in Asian countries where polished rice is the staple food (Thangangkul, 1966). However, the incidence of moderate deficiency has not been studied much in infants and young children. This group may be most at risk for long term effects of thiamin deficiency because such deficiency may impair myelination in the rapidly developing brain (Haas, 1988).

The measurement of transketolase enzyme activity in erythrocytes is currently the most reliable index of thiamin status as it gives an indication of the adequacy of body stores of thiamin (Gibson, 1990). Measurement of the activity of this enzyme in erythrocytes is most frequently used since the erythrocytes are among the first tissues to be affected by thiamin depletion (Brin, 1967). In thiamin deficiency, the basal activity of transketolase is low, and the addition of TPP will produce an enhancement of enzyme activity. The percent increase in activity is known as the "TPP effect". A TPP effect of <15% is considered normal, and > 25% is considered deficient (Brin et al, 1965).

The urinary excretion of thiamin is another commonly used determinant of thiamin status. It is not considered as reliable as the TPP effect as it is only a reflection of immediate intake and may not be a reliable index of tissue stores, distribution or actual

biochemical functioning. The excretion of thiamin decreases proportionately to intake until a critical point after which only variable and minor changes of excretion occur. Thiamin intake, transketolase activity, and urinary excretion of thiamin are known to be related at intakes below 30 $\mu$ g/ kcal (Saubertlich, 1979). The requirement for all age groups, therefore, with a safety factor included, is 40 $\mu$ g/ kcal (Nutrition Recommendations, 1990).

#### **1.4 Fatty acids**

Fat is a critical component in an infant's diet, comprising 50% of energy intake. Besides being a major energy source, it is an almost unlimited form of energy storage, and acts as a vehicle for absorption and transport of fat soluble vitamins. It also supplies fatty acids of the n-3 and n-6 series which cannot be synthesized in mammalian cells. Dietary linolenic (18:3 (n-3)) and linoleic (18:2 (n-6)) acids fulfill these requirements and can be desaturated and elongated to the more bioactive products. These fatty acids are required for normal growth, cell membrane composition and function, and as precursors for important hormonal substances such as prostaglandins and leukotrienes. However, it is important to note that quantitatively (76 % in rats), their major metabolic route is  $\beta$ -oxidation to CO<sub>2</sub> (Cunnane et al, 1995). The central nervous system is 50% lipid, second only to adipose tissue, with most of it structural, composed mainly of the desaturated and elongated products of the essential fatty acids. This predicts an essential functional role in the neural development of the growing infant (Moya,1993).

#### **1.4.1 Fatty acid nomenclature and definitions**

The nomenclature for fatty acids used will be the number of carbon atoms followed by a colon and the number of double bonds. The fatty acid family is identified by the distance of the first double bond from the methyl end ( $n - \# \text{carbons}$ ) of the fatty acid carbon chain. The position where desaturation occurs and the respective desaturase enzyme is designated by the carbon number from the carboxyl end ( $\Delta$ ). The term long chain polyunsaturated fatty acids (LCPUFA) is used to describe fatty acids containing 20 or 22 carbons, and greater than 2 double bonds. The structures and names of the major fatty acids are in figure 1.2.

#### **1.4.2 Metabolism and supply**

##### **1.4.2.1 Endogenous supply**

Fatty acids can originate from various sources. They can be produced de novo from acetyl CoA, which is produced in mitochondria from carbohydrate (via pyruvate oxidation), amino acid degradation, or fatty acid degradation. This biosynthesis occurs in many mammalian cells involving the sequential addition of two carbon units, usually culminating in palmitic acid (16:0). The major pathways of LCPUFA biosynthesis are shown in figure 1.3. Palmitic acid can be further elongated to stearic acid (18:0) in either mitochondria or in microsomes (Rawn, 1989). Alternately, in less common routes, it can be desaturated and elongated or be simply elongated to long chain saturated 20-26 carbon fatty acids (Cook, 1991). Desaturation and elongation takes place on the endoplasmic



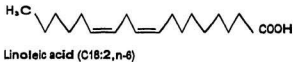
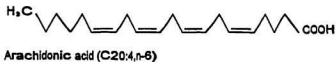
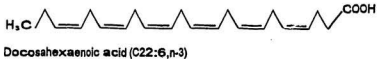
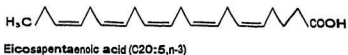
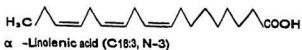
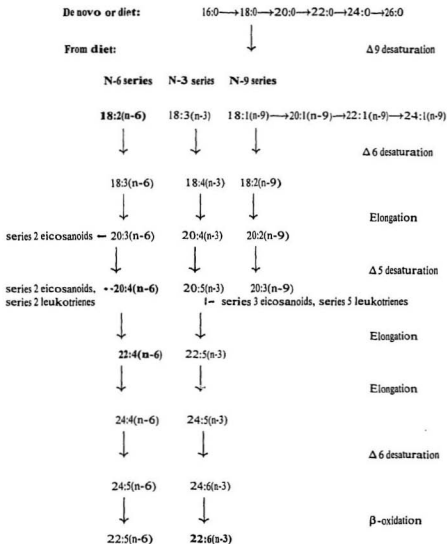


Figure 1.2 Structures of the Major Fatty Acids.



**Figure 1.3 Major Pathways of Fatty Acid Biosynthesis.**

(Adapted from Cook, 1991, Innis, 1992, and Sprecher, 1992)

reticulum membranes of many tissues which contain elongating enzymes, as well as  $\Delta 9$ ,  $\Delta 6$ , and  $\Delta 5$  desaturases. These oxygen-dependent, iron containing desaturases are key amphipathic enzymes assembled in an electron transport system that contains the flavoenzyme NADH-cytochrome  $b_5$  reductase, and cytochrome  $b_5$  (Rawn, 1989).

The  $\Delta 9$  desaturase is the gateway for production of the n-9 family of fatty acids, the only endogenously produced polyunsaturated fatty acids. Stearic acid can be desaturated by this enzyme to oleic acid (18:1(n-9)) and either further elongated and desaturated to eicosatrienoic acid (20:3(n-9)) or just elongated to various minor monounsaturated products. 20:3(n-9) can partially replace for some physical functions of the n-6 and n-3 series within membranes, however it is not a precursor of prostaglandins and cannot alleviate signs of essential fatty acid deficiency (Cook, 1991).

#### **1.4.2.2 Dietary supply**

Many of the above mentioned endogenously produced fatty acids can also be supplied in the diet. However, mammalian cells cannot endogenously produce fatty acids of the n-3 and n-6 series. This is due to the absence of the  $\Delta 12$  and  $\Delta 15$  desaturases required to produce the n-3 and n-6 series of fatty acids. Only plants and insects have this capacity. Fatty acids of these series provide much of the fluid core of cell membranes and are the precursors for eicosanoid and prostaglandin biosynthesis (Cook, 1991).

Therefore, dietary sources are essential and must be provided in the diet primarily as the precursors linoleic acid (18:2(n-6)) and linolenic acid (18:3(n-3)) or their longer chain derivatives. Alternately, the fatty acids, 16:2 (n-6) and 16:3(n-3), found in small amounts

in green vegetables, can be elongated to 18:2(n-6) and 18:3(n-3) respectively (Sprecher, 1968, Cunnane et al, 1995)

For infants, a varying supply of 18:2 (n-6) and 18:3 (n-3) fatty acids is provided in the milk supply. Formulas, which are vegetable oil based, contain only the 18 carbon essential fatty acids. However, animal based milk, such as human milk, also contain the longer chain derivatives (20 and 22 carbon chain). After absorption the fatty acids are incorporated into chylomicrons in the intestine and released into the circulation. The fatty acids are taken up by the liver where they may be elongated and desaturated into other products. Repackaging then occurs into lipoproteins, and they are released into plasma (Bazan et al, 1993). Organs such as the brain, retina, and liver selectively uptake required LCPUFA from the circulation, although they do have limited capacity for synthesis of these fatty acids from the appropriate precursor. Red blood cells, often used as a marker to determine the availability of fatty acids for incorporation into membranes, do not contain the enzymes necessary for elongation and desaturation of fatty acids. However, they incorporate significant amounts of LCPUFA's through the turnover of phospholipid fatty acids. This occurs through deacylation and reacylation of the phospholipid fatty acids from plasma, as well as the much slower exchange of intact phospholipids (Innis, 1992).

#### **1.4.2.3 Regulation of polyunsaturated fatty acid synthesis**

Since n-6, n-9, and n-3 fatty acids all rely on the same enzymes in the microsomal electron transport system, there is competition among potential substrates for a given

desaturase enzyme (Innis,1991). As seen in figure 1.3, the three families of fatty acids all undergo similar reactions, alternating between elongation (the addition of a two carbon unit) and desaturation. 18:1(n-9), formed endogenously, 18:2(n-6), and 18:3(n-3), obtained from the diet, all compete for the  $\Delta 6$  desaturase, which acts at carbon 6-7. Preferential desaturation occurs in the order  $18:3(n-3) > 18:2(n-6) > 18:1(n-9)$  (Brenner,1974). These relative affinities explain the rise in 20:3(n-9) seen in concurrent 18:3 (n-3) and 18:2(n-6) deficiency and its near absence under conditions of adequate essential fatty acid status. As a result of this competition for the same enzyme relatively higher amounts of tissue n-3 derivatives are produced with a low dietary n-3/n-6 ratio, suggesting that the balance as well as absolute amounts of each series affects the ability for synthesis of longer chain derivatives. The rate of  $\Delta 6$  desaturation is also regulated by the concentration of the reaction substrate and product. High amounts of substrate inhibit enzyme activity, whereas low amounts of product induce enzyme activity (Brenner et al, 1966, 1969).

Dietary intake of minerals also is a factor in LCPUFA biosynthesis. Low zinc intake impairs  $\Delta 6$  desaturation, reducing 20:4(n-6) as well as 20:3(n-9) production (Cunnane et al, 1995). As well, high copper intake increases the formation of 20:3(n-9) even with adequate 18:2(n-6) intake (Cunnane, 1985). Therefore a diet which is both low in zinc and copper may result in impaired 20:3(n-9) production.

The  $\Delta 5$  desaturase acts at carbon 5-6, producing eicosapentaenoic acid (EPA-20:5 (n-3) and arachidonic acid (AA-20:4 (n-6)), immediate precursors to the prostaglandins. It has been demonstrated that  $\Delta 5$  desaturation of the respective fatty acid pathways is

inhibited by 18:2(n-6) and 18:3(n-3) (Brenner, 1974). Thus it appears that there is a control mechanism to ensure that excessive amounts of n-3 or n-6 LCPUFA's are not formed in the presence of exceptionally high amounts of 18 carbon precursor.

It was originally assumed that desaturation at the  $\Delta 4$  position occurred through a  $\Delta 4$  desaturase. However, it was recently determined that two elongations from 20 to 22 carbons and 22 and 24 carbon products occurs, followed by  $\Delta 6$  desaturation and  $\beta$ -oxidation in mitochondria and/or peroxisomes to 22:6 (n-3) and 22:5 (n-6) (Sprecher, 1992).

#### **1.4.3 N-6 fatty acids**

The essentiality of linoleic acid was first established in 1929 by Burr and Burr in experiments in which young rats were fed for several months on a fat free diet. Growth retardation, scaly dermatitis, increased transepidermal water loss and reproductive failure were found (Burr, et al, 1929). Later this essentiality was established in human infants by Hansen and others. Infants were maintained for up to several months on diets based on skim milk containing <0.04 to 0.07% of calories from linoleic acid. They quickly developed large stools, and within weeks the skin became dry, thickened and later desquamation with oozing occurred in the intertriginous folds. Dietary additions of linoleic acid or food containing linoleic acid relieved or prevented the condition. Diets based on cow's milk, evaporated whole cow's milk formula, or skim milk with added butterfat did not produce the clinical conditions noted above and actually acted as therapeutic diets for recovery (Hansen et al, 1963). These studies, however, did not

involve intakes in the crucial range of 0.07 and 1.3% kcal as linoleic acid, therefore a more precise cut off of when clinical signs ensue is unknown.

Biochemical indices have also been used to determine the presence of EFA deficiency. Due to the preferential desaturation of n-3 and n-6 fatty acids, rarely do fatty acids of the n-9 series accumulate. However, during n-3 or n-6 deficiency, the accumulation of primarily 20:3 (n-9) occurs, accompanied by a reduced arachidonic acid (20:4 (n-6)) level. Studies determining the fatty acid composition in erythrocytes, plasma and hearts of rats have shown that at intakes falling below about 1% of energy as linoleic acid, the triene:tetraene ratio rapidly increases (Holman, 1960). However, 18:3(n-3) and 18:1(n-9) were also often missing from the diets in these studies, so the specific requirement for 18:2(n-6) is difficult to judge.

Minimal requirements of linoleic acid have also been determined from the amount needed to maintain tissue levels of arachidonic acid in various rat organs. Bourne et al (1990) conducted a study of 21 day old rats in which the level of linolenic acid was kept at 0.4% of calories while the linoleic acid amount was increased. It was found that the liver, lung and kidney had the highest requirement for linoleic acid at 2.4% of energy after which point 20:4(n-6) did not increase further. However, a level of 2.4% as linoleic acid was required to produce maximal accumulation of 22:4(n-6) in the brain.

The importance of an adequate supply of n-6 fatty acids in the diet is demonstrated in autopsy results of infants. These have demonstrated that the n-6 fatty acids continue to be deposited in the forebrain after birth, with arachidonic and adrenic acids the most abundant (Martinez, 1992). A dietary supply of 20:4(n-6) or 22:4(n-6)

does not appear to be a requirement, however, as diets with abundant 18:2(n-6) without a preformed supply produce accumulation in the CNS similar to infants fed breastmilk, which does contain preformed sources (Makrides, 1994, Farquharson, 1992). Autopsy analyses have not been performed on infants fed reduced amounts of linoleic acid; therefore minimal requirements for maximum accretion of n-6 LCPUFA are unknown. The degree to which these fatty acids decrease within organs during a deficiency and any possible functional effects are unknown.

Both the amounts found in breastmilk and triene:tetraene ratios have helped establish the requirements for 18:2 (n-6) to be in the range of 1-4.5% kcal (Nutrition Recommendations, 1990). The 1990 Canadian RNI for infants 0-4 months of age is 4.5 % total energy, based primarily on the average amounts found in breastmilk (Nutrition Recommendations, 1990). In the late 70's an argument was made for values as low as 0.6% kcal, due to the absence of any reported cases of EFA deficiency even when evaporated cow's milk was prevalent (about 0.8% kcal as 18:2(n-6)) (Cuthbertson, 1976). The much higher amounts seen in mother's milk, in combination with what is known about amounts required for optimum structural lipid levels of linoleic or arachidonic acids, membrane associated functions, and eicosanoid metabolism, have indicated that linoleic acid deficiency may be silent, with overt clinical signs only in extreme cases (Innis, 1991).

#### **1.4.4 N-3 fatty acids**

The establishment of  $\alpha$ -linolenic acid as essential for humans has taken much



longer than linoleic acid, due in part to the lack of easily recognizable deficiency symptoms, lack of increase of 20:3 (n-3), the resistance of tissues to n-3 depletion, and the low dietary requirement for n-3 fatty acids (Innis, 1991).  $\alpha$ -Linolenic acid deficiency is mainly of importance during development since, after this time, cases of deficiency have occurred only during very long periods of intravenous nutritional support at extremely low levels of 18:3 (n-3) (Carlson, 1991).

A specific role for 18:3 (n-3) has not been found, however, its elongation and desaturation products, primarily eicosapentaenoic acid (EPA, 20:5 (n-3)) and docosahexaenoic acid (DHA, 22:6 (n-3)) perform many essential roles. EPA is a direct precursor to eicosanoids which help regulate blood clot formation, immune response, and the inflammation response to injury and infection (Whitney et al, 1993). DHA is found in very high levels in central nervous system (CNS) membranes, such as the visual elements of the retina and synaptic terminal membranes (Clandinin et al, 1980).

Diets low in n-3 fatty acids as seen in Western diets is associated with increased risk of heart and inflammatory diseases as a result of the increased production of the more potent 20:4(n-6) derived eicosanoids (Sinclair, 1991). However the significance of low n-3 fatty acids on eicosanoid metabolism in infants is unknown.

DHA (22:6n-3) continues to increase in the brain until at least two years of age. An adequate supply, therefore, of fatty acids from the n-3 fatty acid series is critical for brain development even after birth (Martinez, 1992). The very high amounts (as % total fatty acids) of 22:6(n-3), in particular, present in gray matter of the brain (30%), retina, especially the rod outer segment (38%), and testes, across many species indicates their

importance (Salem, 1985). To determine minimal requirements, the amount of 18:3(n-3) that supports maximal accumulation of n-3 fatty acid derivatives in various organs has been used. In rats, 0.3 to 0.7 % kcal as 18:3(n-3) has produced maximal concentrations of 22:6(n-3) in whole brain lipid, synaptic membranes, retina, and myelin (Bourre et al, 1989). In piglets, 2% of energy as  $\alpha$ -linolenic acid produces adequate accretion of 22:6(n-3) for synaptic membranes and the retina, however formulas with 0.4% of energy as linolenic acid do not, as compared to those fed sow milk. As well, piglets fed formulas with no 22:6(n-3) containing 1.5% versus 0.75% of energy as 18:3(n-3) had higher 22:6(n-3) in the brain and liver (Innis, 1992, Arbuckle et al, 1992, Hrboticky et al, 1990). In human infants, autopsy analysis of CNS tissue has shown that diets with  $\alpha$ -linolenic acid in the range of 0.5-0.8% energy have lower cerebral cortex 22:6(n-3) than infants breastfed, however they have similar retinal composition (Makrides et al, 1994, Farquharson et al, 1992).

Functional assessment in rats, primates and both preterm and full term infants of dietary n-3 adequacy has been done primarily through measurements of visual system development. A link between vision and n-3 fatty acid deficiency was first observed when a young patient on total parenteral nutrition (TPN) experienced blurred vision (Holman et al, 1982). The relationship of dietary intake of n-3 fatty acids, organ accretion, and visual development was then established in infant rhesus monkeys by Neuringer et al (1984, 1986). Infant rhesus monkeys on diets very high in linoleic acid (75% total fatty acids), but low in  $\alpha$ -linolenic acid (0.3% total fatty acids), had decreased accretion rates of n-3 derivatives, altered electroretinographic responses, and reduced

visual acuity.

These functional effects are explained by DHA's critical role in the visual process. The highest levels of 22:6(n-3) in the body (50 mol %) are found in the disk membranes of the outer segments of photoreceptor cells. These cells are the site of vision initiation, and are packed with visual pigment. The rods contain the pigment rhodopsin which appears to require specifically the presence of 22:6(n-3) for optical activity, as phospholipids containing 22:6(n-3) associate with rhodopsin strongly (Neuringer, 1993, Deese et al, 1981).

Methods commonly used to measure visual function are visual acuity or electroretinograms. Visual acuity measures cortical function and electroretinography measures retinal effects. Electroretinograms measure the electrical signal that the photoreceptor cells emit when stimulated by light. The most commonly used methods of visual acuity are visual-evoked potential acuity (VEP) and preferential looking acuity (PL). VEP testing involves the response of the infant measured by electrodes to changes in visual patterns on a video screen. PL, most commonly using the Teller acuity card procedure, involves showing an infant a set of cards with stripes of varying width on one side and a blank screen on the other. By observing the looking behaviour of the infant, the examiner determines which side the stripes are on (Teller, 1988). These visual acuity tests are based on the inherent tendency for infants to gaze at a discriminable pattern rather than a blank screen, and when the infant can no longer discern a pattern, it is considered to be their acuity score (Neuringer, 1993).

Studies on preterm infants consuming diets lacking 22:6(n-3) but with reasonable

amounts of 18:3(n-3) have consistently demonstrated a decrease in visual function as compared with infants fed marine-oil based formulas or breastmilk which contain preformed 22:6(n-3) (Uauy et al, 1992, Carlson et al, 1993). It thus appears that 22:6(n-3) is essential in preterm infants. Preterm infants, however, in contrast to those born fullterm, are particularly at risk due to the rapid accretion of 22:6(n-3) in the retina and CNS during the last trimester of pregnancy (Martinez, 1992). In fullterm infants, whether preformed 22:6(n-3) is required in the diet to produce optimal visual function as well or if adequate amounts of its precursor 18:3(n-3) can fulfill the requirements has produced mixed results, depending on the level of 18:3(n-3) as well as the type of visual testing being performed.(Makrides, 1993, Innis, 1994, Birch, 1992). Research has shown that full term infants fed formulas without preformed 22:6 (n-3) containing 18:3(n-3) as 1% of energy could match the visual function of breastfed infants (Innis, 1994), however, 0.4% (Birch, 1992), or 0.5-0.8% (Makrides, 1993) of energy as 18:3(n-3) does not. Therefore, more research must be done to clarify whether adequate 18:3 (n-3) is enough to fulfill the CNS requirements for n-3 LCPUFA's.

## CHAPTER 2.0      PROBLEM STATEMENT

From the 1930's to the late 60's the feeding of evaporated milk formulas in North America was prevalent (Cone, 1981). Although evaporated milk usage has dropped worldwide, it remains common in Newfoundland, Canada, due to both traditional and economic reasons (Matthews et al, 1992). Evaporated milk formula, although adequate for energy, contains excess protein and less fat than formula or breastmilk. Evaporated milk also is low in iron, copper, zinc, selenium, vitamins B<sub>1</sub>, K, and A and both essential fatty acids, linoleic and  $\alpha$ -linolenic (Nestle, 1991, Litov, 1991, Sanders et al, 1979).

Thiamin, in particular, in diluted evaporated milk (1:2 dilution) contains only 0.30 mg/1000 kcal compared to the recommended intake of 0.40 mg/1000 kcal for infants (Nestle, 1991). Breastmilk, on average, contains only 0.22 mg/1000 kcal (Health and Welfare Canada, 1987). As there are very few studies on thiamin status in infancy this recommendation is based more on adult data than infant. Studies which have been done did not report dietary intakes of thiamin or type of feeding. Thiamin's role in energy metabolism and neurological development emphasizes the importance in determining its adequacy in various diets.

There has been a resurgence of interest in recent years of the fatty acid requirements of infants which has led to a reexamination of the adequacy of the fatty acid composition in various formulas. This has been led by the discovery that the circulating fatty acid composition in erythrocytes and plasma is known to differ in infants either breastfed or fed formulas of differing fatty acid composition (Innis, 1991). As well,

vision has been known to be affected in infants fed inadequate quantities of essential fatty acids (Makrides et al, 1994, Uauy et al, 1992). Breastmilk contains both the essential fatty acids and their long chain polyunsaturated products (LCPUFA's), commercial formulas contain essential fatty acids but do not contain LCPUFA's (Innis, 1991). LCPUFA's are found in very low amounts in evaporated milk (Sanders et al, 1979)). The central nervous system (CNS) accretes high amounts of LCPUFA's during the brain growth spurt which continues until 18 months of age, indicating that an adequate supply in infants is critical (Clandinin et al, 1980).

The present study, therefore, sought to compare the effects of feeding breastmilk, commercial formulas, or evaporated milk on growth, thiamin status, and fatty acid status in healthy, fullterm infants.

The objectives of the present study were:

- 1) to observe and compare the growth patterns of all groups by determining length, weight, and head circumference
- 2) to determine the adequacy of thiamin intakes of all groups by:
  - a) comparison of actual intakes with recommended intakes
  - b) measuring thiamin status through erythrocyte transketolase activity and the thiamin pyrophosphate effect, a functional assay of thiamin adequacy
- 3) to determine the adequacy of the fatty acid content for infants by:
  - a) comparing the fatty acid composition of all milk and formula consumed
  - b) comparing the fatty acid composition in the phosphatidylethanolamine fraction of erythrocytes

- c) comparing the performance on a functional test of fatty acid adequacy (visual acuity).

## **CHAPTER 3.0      METHODS**

### **3.1                Subjects**

Healthy, fullterm infants were recruited from either the Grace General hospital in St. John's or through public health nurses in surrounding areas. All infants were eligible if they weighed between 2500 - 4500 grams at birth, were of 38-42 weeks gestation, had no health problems or anomalies, and informed parental consent was obtained (appendix A). Between January and October of 1993, mothers of eligible infants were approached before discharge from hospital by the research nurse. The planned method of feeding was then determined with no recommendations or advice given. Those with an intention to either feed human milk (BM), the commercial formula's Similac® or Enfalac® (F), or an evaporated milk formula (EM) exclusively for the duration of the study (six months) were asked to take part. For attendance at each of the three and six month clinics, the mothers received a \$40 stipend to help offset costs such as travel or child care.

Subjects were recruited in consecutive order until approximately 35 infants were recruited in each group. Due to switching of feed groups, moving, or travel difficulties, some subjects were lost from follow-up. At three months the enrollment was BM (n=35), F (n=34), and EM (n=31), with a final enrollment of 30 in each group at six months. As this study is one part of a larger study on the overall nutritional status of these infants, an adequate amount of blood could not always be obtained for all tests, therefore there were incomplete data sets for some infants.



### **3.2 Protocol**

At baseline (<3 days of age) a blood sample was obtained via a heel prick before discharge at the Grace General Hospital and weight, length, and head circumferences were obtained from hospital records. At three and six months of age ( $\pm 14$  days) the subjects attended a research clinic at the Janeway Child Health Centre in St. John's or the Carbonear General Hospital where a blood sample was taken via venipuncture. Weight, length, and head circumference were measured, visual acuity testing was performed, and the dietary record was reviewed. A milk sample was obtained from all mothers still breastfeeding at the three and six months clinics and a formula sample from the others at the three month clinic. As well, a questionnaire regarding parental information such as maternal age, education, and socioeconomic status was completed at the three month clinic (appendix B). During the study mothers were telephoned at biweekly intervals by the research nurse to determine compliance. As well, before each of the three and six month clinics, the mothers were provided information in writing about the clinic appointment, the milk sample, and how to fill out the dietary record (appendix C). All blood samples were taken in cooperation with staff laboratory technologists at the respective hospitals and visual acuity testing was performed in cooperation with Dr. Mary Courage (Department of Psychology). The protocol and procedures were approved by all hospital and university Ethics Committees.

### **3.3 Dietary intakes**

Dietary intake was obtained from three day dietary records. These were mailed to

the mother and contained instructions on how to record food intake, with columns for amount consumed, brand names of foods, and time of feeding. They were asked to fill these out in the last three days before the clinic visit. At the clinic, the diet record was briefly reviewed and any inconsistencies were clarified. The timing of solid food or supplementary milk introduction was also determined. If the food record was not completed, a 24 hour recall was performed. The main feed, any vitamin or mineral supplements, solid foods or supplemental formula were recorded. All intake was converted to grams, assigned a food code, and entered onto the computer database (Friel et al, 1985). The database was updated with nutrient information obtained from the various formula and food companies. As the volume of breastmilk intake was not determined, intake was based on an estimation of milk consumption of 750 ml/day at three months. At six months of age, based on data from partially and exclusively breastfed infants, exclusively breastfed infants (>90% estimated energy from breastmilk) were estimated to consume on average 77 kcal/kg, and those who supplemented were estimated to consume 81 kcal/kg (Hienig et al, 1994). Energy intake was calculated on a per kilogram basis and energy from solid food or formulas were subtracted from the total, with the remainder of intake attributed to breastmilk. The energy intake from breastmilk was converted to the equivalent grams and was entered onto the computer so that an estimate of contribution of breastmilk for nutrient intake could be determined. The fatty acid composition was determined from analysing milk samples obtained from each subject in all groups at the three month clinic as well as those still breastfeeding at the six month clinic.

### **3.4 Anthropometry**

All anthropometric measurements were done in triplicate by the same two examiners. Recumbent length was measured using an in-house infantometer (Memorial University Technical Services) to the nearest millimeter. Weight was determined using a pediatric spring scale with a pan. A towel was placed on the scale and the weight was taken to the nearest gram when the infant was lying quietly. Calibration was done at the start of each clinic using a 5 kg weight. Head circumference was measured using non-stretch tape (Ross Laboratories, Columbus, Ohio). Z scores for length for age, weight for age, and weight for length were calculated using CASP (Anthropometric Software Program, version 3.0, 1987, Division of Nutrition, Center for Health Promotion and Education, Centers for Disease Control, Atlanta, Ga). The calculation is as follows:  $(\text{Actual anthropometric value} - \text{median reference value}) \div \text{Standard deviation (S.D.)}$ . Reference values were those obtained from the National Center for Health Statistics growth data (Hamill et al, 1979).

### **3.5 Visual acuity**

Binocular preferential looking (visual) acuity was tested using the Teller acuity card procedure. This test is based on the inherent tendency for infants to gaze at a discriminable pattern rather than a blank screen and has been described in detail elsewhere (Teller et al, 1988). The Teller Acuity Cards (Vistech Inc.) consist of a set of 16 rectangular gray cards, 15 of which contain a black and white square grating (stripes) embedded in a luminance-matched gray background, which is to the left or right of a

small central peephole. A trained observer, positioned 55 cm from the infant, showed the subject the series of cards beginning with the grating of lowest spatial frequency (i.e., the widest stripes) of 0.3 cycles/degree and advancing in half-octave steps from coarse to fine gratings. The tester, who was unaware of which side contained the gratings, determined it by observing the infant's looking behaviour through the peephole. The finest grating resolved determined the visual acuity score.

### **3.6 Sample collection**

#### **3.6.1 Milk**

Formula or breastmilk samples were obtained from all mothers at the three month clinic and from the mothers who were continuing to breastfeed at the six month clinic. A 15 ml polypropylene screw-top vial (Corning Centrifuge) was mailed with the diet record for all breastfeeding mothers. The mothers were asked to hand express a sample, keep it in the refrigerator without freezing, and bring it to the clinic. Samples of milk from the remaining infants was poured off directly from the current bottle the infant had been fed. For all samples approximately three mls was placed in screw top glass vials and placed on ice, nitrogen gas was blown over it, and the samples were frozen at  $-70^{\circ}\text{C}$  until analysis.

#### **3.6.2 Blood**

At baseline, 500-800  $\mu\text{l}$  of blood was taken via a heel prick and collected in microtainers (Becton Dickinson). At three and six month collections, approximately two

to three millilitre (ml) of blood was taken from the infant's arm by venipuncture and drawn into vacutainers (Becton Dickinson) containing EDTA as anticoagulant. Baseline blood samples were placed on ice and transported immediately to the university laboratory for separation, washing, and aliquoting. However, blood samples obtained at the three and six month clinics were handled in 4°C cold rooms on site. Blood samples were centrifuged (5415 Eppendorf centrifuge, Brinkman) at 4°C at 3-4000 rpm, plasma was removed, and the red cells were washed three to four times in ice cold saline at 4°C. The blood was then immediately aliquoted for various tests. For the thiamin assay, 200-400 µl of erythrocytes were diluted with equal amounts of distilled water and stored in plastic eppendorf tubes. For the fatty acid assay, 100 to 300 µl of erythrocytes were placed in 4 ml glass screw-top vials and blown over with nitrogen gas. All samples were stored at -70°C.

### **3.7 Fatty acid analyses**

#### **3.7.1 Erythrocyte lipid**

Comparative analyses of the fatty acid composition of red blood cell and plasma glycerophospholipids among infants fed various formulas and breastmilk have been used as a measure of the adequacy of essential fatty acids (Innis, 1991). In the present study, the fatty acid composition of the phosphatidylethanolamine (PE) fraction of erythrocytes was determined. The PE fraction was used as it is concentrated primarily on the inner part of the red cell membrane and should therefore be less prone to dietary fluctuations

and theoretically provide a better index of organ fatty acid composition.

The steps in the analyses briefly involve: extraction of lipid from washed erythrocytes, separation of phospholipids by thin layer chromatography, methylation of the fatty acids, and subsequent quantification and identification of relative amounts of fatty acids by gas-liquid-chromatography. The fatty acid composition of the various formulas and breastmilk was also determined, similar to erythrocytes, however, total lipid was used for analyses.

The steps in erythrocyte fatty acid analyses are as follows:

i) *Extraction.* Red blood cell lipids were extracted by the method of Rose and Oklander (Rose et al, 1965). An equal volume of distilled water was added to the red cells, vortexed, and allowed to stand for 15 minutes. Isopropanol (HPLC grade) as eleven times the red cell volume (11:1) was added slowly with occasional vortexing over an hour. Chloroform (HPLC grade) as seven times the original red cell volume was added, the sample was vortexed, and allowed to stand for one hour. The sample was then centrifuged for five minutes at high speed and the extract was poured off into 100 ml cylinders. The extract was washed sequentially with twenty times the volume as 2:1 chloroform: methanol and 0.2 the volume as 0.37% KCl, followed by 0.2 the volume as methanol/water (1:1, v/v) (Folch, 1957). Each time the cylinder was inverted and then allowed to stand until layer separation occurred. The top aqueous layer was then removed by aspiration. The lipid extract was dried under nitrogen gas and redissolved in 50  $\mu$ l of chloroform:methanol (9:1).

ii) *Thin Layer Chromatography.* Individual phospholipid classes were resolved

by the thin layer chromatography method of Shipky et al, (1964) (chloroform, methanol, acetic acid, and water, 25:15:4:2 by volume) on silica Gel G plates and spots were identified by comparison with commercial phospholipid standards (Sigma Chemical Co.). The bands were visualized by iodine vapour, and the phosphatidylethanolamine (PE) band was scraped onto wax paper and transferred into test tubes.

iii) *Elution*. The phospholipid was eluted from the silica by adding 2 ml of 9:1 chloroform/ methanol, vortexing, and centrifuging for 10 minutes and pouring off the supernatant into transmethylation vials (Supelco). This was repeated three times in order to recover all the phospholipid.

iv) *Transmethylation*. The phospholipid extract was evaporated to dryness and the fatty acids were methylated by the addition of 1.5 ml of 94:6 methanol:HCL and placed in a 65°C oven for 15 hr with hydroquinone as an antioxidant.

v) *Recovery of methyl esters*. The fatty acid methyl esters (FAME's) were recovered from the methanol /HCL solution by adding 1.5 ml of hexane, and drawing off the upper (organic) layer. This was performed three times to ensure maximum sample recovery. Water (1.5 ml) was then added and the hexane layer containing the FAME's was again drawn off. This step was repeated twice and was followed by placing the samples in a -20°C freezer, to freeze any remaining water. The sample was poured off and evaporated to dryness.

vi) *Gas-liquid-chromatography*. The methyl esters were dissolved in CS<sub>2</sub>, and placed in insert vials. Fatty acid methyl esters were separated by gas-liquid-chromatography using a Supelcowax 30 m capillary column in a Hewlett Packard 5890

Series 11 GC. Oven temperature was 190°C, ramped to 220°C at 15 min at a rate of 5°C/min for 12 min and the injection port and flame ionization detector temperatures were 230°C. Identifications of fatty acids were determined by comparing retention times with authentic standards and quantified by total weight percent (Nu-Chek-Prep Inc., Supelco Inc.). Integration of peak areas was done by Hewlett Packard 3365 Series 11 Chemstation software. Peaks that could not be identified were discounted from the area percent. Therefore, the weight percent calculated was of total identified fatty acids.

### **3.7.2 Milk**

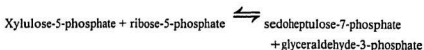
Before lipid extraction, breastmilk samples were heat treated in a 80°C water bath for 90 seconds to inactivate lipase activity (Bitman et al, 1984). Lipids from formulas and breastmilk were extracted with chloroform:methanol (2:1) by suction filtration to remove precipitate. Fatty acid methyl esters were then formed directly from the lipid extract and the remainder of the preparation was as for erythrocytes, mentioned above. As >98% of the peaks could be identified as known fatty acids, the relative weight percents were not recalculated. Breastmilk samples were analyzed from 29/35 mothers. Representative samples of the other formulas were also analyzed (EM, n=6, Similac<sup>®</sup>, n=5, Enfalac<sup>®</sup>, n=3).

### **3.8 Thiamin assay**

Erythrocyte transketolase activity and the thiamin pyrophosphate effect (TPPe) was measured by the method of Brin et al, (1965) to serve as a functional evaluation of



thiamin adequacy. Transketolase, an enzyme of the pentose phosphate pathway, is dependent on the active form of thiamin, thiamin pyrophosphate (TPP). In the red blood cells, as thiamin reserves become depleted, transketolase activity is reduced, and is recovered only through the provision of TPP. The test is based on the following reaction which requires transketolase bound to thiamin pyrophosphate:



The disappearance of the added substrate, ribose-5-phosphate (transketolase substrate in pentose phosphate pathway), at pH 7.4 and 37°C gives a measure of transketolase activity in µg pentose utilized/ml hemolysate/hour. The percent increase in activity of red cells saturated with added TPP compared to those with only endogenous TPP gives an indication of thiamin adequacy in the tissues, called the TPP effect (Brin et al, 1965).

Initially, the hemolysate was preincubated in a buffered medium with or without added TPP for 30 min, in order for the coenzyme (TPP) to attach to transketolase. It was then incubated with an excess of substrate (ribose-5-phosphate) for 60 min at 37°C, with trichloroacetic acid (TCA) used to stop the reaction and denature the protein (incubation chart, table 3.1). The samples were then centrifuged and the protein-free filtrate was used to determine the amount of pentose utilized. The pentoses remaining, which consisted of an equilibrium mixture of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate, were determined by a colorimetric assay (table 3.2). In this assay, these

**Table 3.1 Incubation chart for determination of erythrocyte transketolase activity and thiamin pyrophosphate effect.**

<b>Tubes</b>	<b>Hemolysate (ml)</b>	<b>Buffer (ml)</b>	<b>TPP (ml)</b>	<b>Pre-incubation time (min) (37°C)</b>	<b>Substrate (R-5-P) (ml)</b>	<b>Incubation time (min) (37°C)</b>	<b>5% TCA (ml)</b>
<b>A</b>	0.1	0.4	0.0	30	0.1	60	3.0
<b>B</b>	0.1	0.0	0.4	30	0.1	Vortex	3.0
<b>D</b>	0.1	0.5	0.0	30	0.0	60	3.0
<b>R</b>	0.0	0.5	0.0	---	0.1	60	3.0

**Table 3.2 Determination of pentose utilization in transketolase assays.**

<b>Tubes</b>	<b>Filtrate (ml)</b>	<b>Pentose (ml)</b>	<b>Distilled H<sub>2</sub>O (ml)</b>	<b>Orcinol (ml)</b>	<b>Boiling Water Bath</b>	<b>Cold water bath</b>
<b>A, B, and D</b>	0.2	0.0	1.3	4.5	1	1
<b>R</b>	0.1	0.0	1.4	4.5	1	1
<b>5 standard</b>	0.0	0.5	1.0	4.5	Vortex 20 min	5 min
<b>10 standard</b>	0.0	1.0	0.5	4.5	1	1
<b>Blank</b>	0.0	0.0	1.5	4.5	1	1

pentoses were converted to furfural and furfural derivatives by concentrated HCl and the condensation of a polyhydric phenol (orcinol) in the presence of metal ions ( $Fe^{+++}$ ) to form colour complexes. These compounds have an absorption maxima at 670 nm and the absorbances were read against a blank set at zero in a Milton Roy Spectronic 601 spectrophotometer.

The standards determine the optical density per microgram of pentose and from this the amount of pentose in the other tubes can be calculated. The calculation in appendix D determines the micrograms pentose utilized per milliliter per hour (transketolase activity). By comparing the relative activity of transketolase with or without the addition of TPP, the TPP effect can be determined. Ranges for thiamin adequacy used were those established by Brin et al (1965):

Thiamin condition	TPP Effect
Normal	<15%
Marginal	15-25%
Deficient	>25%

### 3.9 Statistical analyses

The diet effects at each time were analyzed by oneway analysis of variance (ANOVA) with the least significant difference test (LSD) as the post-hoc test. The fatty acid composition data was arcsine transformed before ANOVA to correct for the non-normal distribution of percentage data (Zar, 1984). The effect of time was determined by a series of paired t-tests, using a Bonferroni correction for the number of comparisons.

Regression analysis was used to determine correlations between variables. All biochemical and growth data was analysed using SPSSx. Visual acuity was analysed using repeated measures analyses of variance with Neuman-Keuls as the post-hoc test. Statistical significance for all analyses was assigned to  $p < 0.05$ .

## **CHAPTER 4.0      RESULTS**

### **4.1                Subjects**

Subject group characteristics are presented in table 4.1. The maternal age of those feeding EM was significantly younger than either other group. As well, breastfeeding mothers averaged the highest in education and socioeconomic indices, followed by formula feeding mothers, then mothers feeding EM. There was no differences in number of children between feed groups.

### **4.2                Dietary intakes**

Of the breastfed infants, at three months, 29 of the 35 infants were exclusively breastfed. Five infants were receiving supplemental formula ranging from 5 to 30% energy. Two infants were receiving both cereal (< 1% energy) and supplemental formula. One infant was receiving <1% of energy as cereal and no supplemental formula. In the F group, at three months of age, 29 infants were fed Similac<sup>®</sup>, and five infants were fed Enfalac<sup>®</sup>. All were fed formula from birth. Twelve infants were receiving solid foods comprising up to 4% of energy, primarily as cereal. In the EM group, 16 infants were consuming solid foods, ranging from <1% to 19% of energy, primarily as custard and cereal. Three infants in the EM group consumed commercial formula for the first two to three weeks, before switching exclusively to EM.

At six months, in the BM group, 9 of 30 infants had been weaned between 3 1/2 and 5 1/2 months, with four infants exclusively breastfed. Eleven infants consumed

**Table 4.1 Characteristics of subject groups.**

	Baseline - 3 months			6 months		
	BM	F	EM	BM	F	EM
<b>Subject Number (n)</b>	35	34	31	30	30	30
<b>Sex M</b>	18	17	22	14	17	21
<b>F</b>	17	17	9	16	13	9
<b>Maternal age (yrs)</b>	29.5 ± 4.2 <sup>a</sup>	27.7 ± 4.7 <sup>a</sup>	24.5 ± 5.3 <sup>b</sup>			
<b>Socioeconomic Index<sup>1</sup></b>	41.3 ± 18.6 <sup>a</sup>	33.8 ± 16.8 <sup>b</sup>	22.2 ± 7.9 <sup>c</sup>			
<b>Maternal Education<sup>2</sup></b>	6.2 ± 1.7 <sup>a</sup>	5.1 ± 1.9 <sup>b</sup>	3.6 ± 1.3 <sup>c</sup>			
<b># Children</b>	2.3 ± 1.9	1.9 ± 0.9	2.2 ± 1.2			

Values with different superscripts in any row are significantly different ( $\pm$  SD) ( $p < 0.05$ ).

<sup>1,2</sup> See appendix B for explanation.

greater than 2/3 of energy from breastmilk. The remaining 6 infants consumed 10-57% energy as breastmilk. Nine mothers were supplementing breastmilk with commercial formula. Solid food intakes accounted for an average of 15% of energy (<1-48%). In the F group, an average of 18% of energy was from solid foods (2-42%). Four infants were consuming Enfalac® at 6 months and the remaining 26 were consuming Similac®. Infants fed evaporated milk consumed on average 23% (3-49%) of energy as solid food. The timing of solid food introduction was 12.2, 14.5 and 18.7 weeks for EM, F, and BM, respectively, and all groups were significantly different.

The dietary intakes of protein, fat, and energy are presented in table 4.2. Energy and protein intakes at three and six months were significantly lower in the breastfed infants. Protein intakes were in the order, EM > F > BM ( $p < 0.05$ ) for both three and six months of age. EM diets were lower in fat than both formula and breastfed infants at three months, and at six months formula fed infants had higher fat intakes. It must be taken into consideration that breastmilk values were volume estimates based on age and weight of the infant with nutrient values determined by the nutrient database.

#### **4.3 Growth**

There were no differences in weight, length or head circumference between feed groups at any time period (tables 4.3-4.5). Weight-for-age, length-for-age, and weight-for-length  $z$  scores did not differ between groups, either (table 4.6). The weight-for-age  $z$  scores were close to one half a standard deviation above the NCHS median at three



**Table 4.2 Macronutrient Intakes per Day ( $\bar{x} \pm \text{SD}$ )**

	<b>BM</b>	<b>F</b>	<b>EM</b>
<b>Energy (Kcal)</b>			
3 Months	531 $\pm$ 35 <sup>a</sup>	642 $\pm$ 97 <sup>b</sup>	637 $\pm$ 163 <sup>b</sup>
6 Months	636 $\pm$ 110 <sup>a</sup>	827 $\pm$ 106 <sup>b</sup>	756 $\pm$ 184 <sup>b</sup>
<b>Protein (g)</b>			
3 Months	9.2 $\pm$ 0.8 <sup>a</sup>	15.1 $\pm$ 2.2 <sup>b</sup>	26.1 $\pm$ 7.7 <sup>c</sup>
6 Months	14 $\pm$ 6.0 <sup>a</sup>	20.3 $\pm$ 3.1 <sup>b</sup>	30.4 $\pm$ 9.9 <sup>c</sup>
<b>Fat (g)</b>			
3 Months	33.2 $\pm$ 1.9 <sup>a</sup>	34.5 $\pm$ 5.0 <sup>a</sup>	28.8 $\pm$ 8.5 <sup>b</sup>
6 Months	32.7 $\pm$ 6.3 <sup>a</sup>	38.5 $\pm$ 6.0 <sup>b</sup>	31.1 $\pm$ 10.5 <sup>a</sup>

Values with different superscripts in any row are significantly different at that time period ( $p < 0.05$ )

**Table 4.3 Weight and weight gain velocity ( $\bar{x} \pm SD$ ).**

<b>Feeding Group</b>	<b>Birth weight (g)</b>	<b>3 Months (g)</b>	<b>6 Months (g)</b>	<b>Weight gain (0- 3mos) (g/day)</b>	<b>Weight gain (3 - 6 mos) (g/day)</b>
<b>BM</b>	3554 $\pm$ 334	6097 $\pm$ 686	7505 $\pm$ 870	28.25 $\pm$ 7.24	15.52 $\pm$ 3.97 <sup>a</sup>
<b>F</b>	3574 $\pm$ 401	6061 $\pm$ 642	7870 $\pm$ 921	27.62 $\pm$ 6.06	20.43 $\pm$ 5.76 <sup>b</sup>
<b>EM</b>	3562 $\pm$ 441	6090 $\pm$ 736	7941 $\pm$ 1166	28.08 $\pm$ 7.51	20.21 $\pm$ 6.79 <sup>b</sup>

Values with different superscripts in any column are significantly different at that time period ( $p < 0.05$ )

**Table 4.4 Length and length gain velocity ( $\pm$  SD).**

	baseline (cm)	3 months (cm)	6 months (cm)	0-3 months (cm/day)	3-6 months (cm/day)
BF	50.90 $\pm$ 1.28	61.42 $\pm$ 1.65	67.43 $\pm$ 2.05	0.12 $\pm$ 0.01	0.07 $\pm$ 0.01
F	50.88 $\pm$ 1.99	61.44 $\pm$ 2.33	67.94 $\pm$ 2.51	0.12 $\pm$ 0.01	0.07 $\pm$ 0.01
EM	50.73 $\pm$ 2.31	61.25 $\pm$ 2.07	67.64 $\pm$ 2.19	0.12 $\pm$ 0.02	0.07 $\pm$ 0.01

There was no differences between groups at any time period.

**Table 4.5 Head circumference and head circumference growth velocity ( $\pm$  SD).**

	<b>baseline (cm)</b>	<b>3 months (cm)</b>	<b>6 months (cm)</b>	<b>0-3 Months (cm/day)</b>	<b>3-6 Months (cm/day)</b>
<b>BF</b>	35.00 $\pm$ 1.13	40.87 $\pm$ 1.19	43.19 $\pm$ 1.16	0.07 $\pm$ 0.01	0.027 $\pm$ 0.00 <sup>a</sup>
<b>F</b>	35.33 $\pm$ 1.17	41.06 $\pm$ 1.26	43.97 $\pm$ 1.41	0.06 $\pm$ 0.01	0.031 $\pm$ 0.01 <sup>b</sup>
<b>EM</b>	35.10 $\pm$ 1.26	40.66 $\pm$ 1.31	43.42 $\pm$ 1.48	0.06 $\pm$ 0.01	0.032 $\pm$ 0.01 <sup>b</sup>

Values with different superscripts in any column are significantly different at that time period ( $p < 0.05$ )

#### 4.6 Z Scores ( $\pm$ SD).

	Z weight-for-age		Z height-for-age		Z weight-for-height	
	3 months	6 months	3 months	6 months	3 months	6 months
<b>BM</b>	0.53 $\pm$ 0.81	0.10 $\pm$ 0.91	0.45 $\pm$ 0.75	0.35 $\pm$ 0.72	0.10 $\pm$ 0.77	-0.32 $\pm$ 0.84
<b>F</b>	0.51 $\pm$ 0.68	0.44 $\pm$ 0.88	0.49 $\pm$ 0.84	0.50 $\pm$ 0.80	0.05 $\pm$ 0.68	-0.05 $\pm$ 0.80
<b>EM</b>	0.41 $\pm$ 0.77	0.43 $\pm$ 1.12	0.33 $\pm$ 0.80	0.30 $\pm$ 0.82	0.12 $\pm$ 0.75	0.11 $\pm$ 1.15
<b>Overall</b>	0.49 $\pm$ 0.75	0.32 $\pm$ 0.98	0.43 $\pm$ 0.75	0.39 $\pm$ 0.78	0.09 $\pm$ 0.73	-0.09 $\pm$ 0.95

months for all groups, as well as at six months for F and EM groups. The BM group demonstrated a decrease in weight gain velocity at 6 months, dropping to just slightly above the median for weight-for-age. Length-for-age z-scores were in the range of a third to one half a standard deviation above the median for all groups at three and six months. The weight-for-height z scores were closer to the NCHS median, however, at six months the BM group fell substantially below. Between three and six months of age, the average weight and head circumference daily increase was significantly lower in the breastfed infants than the other two groups (tables 4.3-4.5).

#### **4.4 Thiamin**

##### **4.4.1 Dietary intakes**

Dietary intake of thiamin expressed as both total and per energy intake is presented in table 4.7, as well as the percent of infants with intakes below the recommended intake (RNI) of 0.40 mg/1000 kcal (Nutrition Recommendations, 1990). The thiamin intakes of both the breastfed and EM fed infants are quite low, with a substantial number of infants below the RNI. However, thiamin content in breastmilk was not determined and can vary from subject to subject. Commercial formulas are well fortified with thiamin, and all intakes were in excess of recommended amounts.

##### **4.4.2 Transketolase activity and tpp effect**

Transketolase activity in breastfed infants was significantly lower at three months

**Table 4.7 Thiamin intakes ( $\bar{x} \pm \text{SD}$ ).**

<b>Age of Infants</b>	<b>BM</b>	<b>F</b>	<b>EM</b>
<b>3 months (mg/day)</b>	0.13 $\pm$ 0.04	0.64 $\pm$ 0.13	0.24 $\pm$ 0.25
<b>mg/1000 kcal</b>	0.25 $\pm$ 0.05	0.99 $\pm$ 0.08	0.38 $\pm$ 0.39
<b>% &lt; RNI</b>	(94%)	(0%)	(68%)
<b>6 months (mg/day)</b>	0.56 $\pm$ 0.34	1.03 $\pm$ 0.29	0.48 $\pm$ 0.29
<b>mg/1000 kcal</b>	0.88 $\pm$ 0.52	1.24 $\pm$ 0.32	0.66 $\pm$ 0.38
<b>% &lt; RNI</b>	(21%)	(0%)	(28%)

of age than the formula fed infants (table 4.8, figure 4.1). This, however was not reflected in a higher thiamin pyrophosphate effect (TPPe) as there were no differences between groups at any time period (table 4.9, figure 4.2).

Transketolase activity decreased with time for all groups. For the TPPe, only the BM group demonstrated a change with time, with an increase from baseline to three months. The percent in each feed group with a TPPe above normal (>15%) was 16% (BM), 13% (F) and 11% (EM). There were no differences between groups in transketolase activity at six months of age. Three infants, all in the EM fed group, had a TPPe value above normal.

Neither the transketolase activity nor the TPPe correlated with weight, weight gain, or dietary intake of thiamin at any time period. However, transketolase activity positively correlated with energy intake at three months of age ( $r = 0.28$ ,  $p = 0.004$ ).

## **4.5 Fatty acids**

### **4.5.1 Milk**

Table 4.10 shows the mean percent by weight of total fatty acids in the breastmilk at three and six months, the commercial formulas (Similac® and Enfalac®) and evaporated milk.

Evaporated milk fat was the most saturated, at 61% of total fatty acids, followed by the commercial formulas, at 46%, and breastmilk, at 37%. However breastmilk contains the most monounsaturates (44%), followed by Enfalac® (34%), EM (26%), and

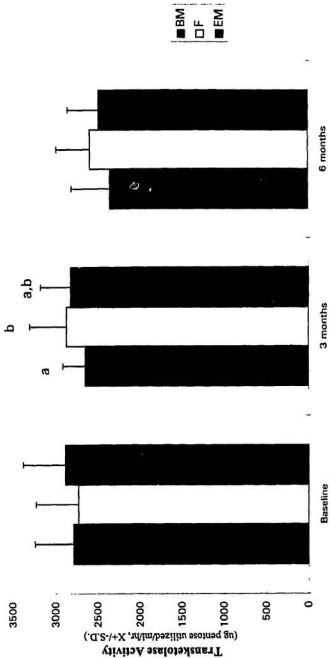


**Table 4.8 Transketolase activity ( $\mu\text{g}$  pentose utilized/ml hemolysate/hour) ( $\bar{x} \pm \text{SD}$ ).**

	<b>Baseline</b>	<b>3 Months</b>	<b>6 months</b>
<b>BM</b>	2792 $\pm$ 457 <sup>1</sup> (n=34)	2653 $\pm$ 269 <sup>a,1,2</sup> (n=34)	2358 $\pm$ 464 <sup>2</sup> (n=25)
<b>F</b>	2733 $\pm$ 503 <sup>1,2</sup> (n=31)	2880 $\pm$ 435 <sup>b,1</sup> (n=32)	2602 $\pm$ 399 <sup>2</sup> (n=25)
<b>EM</b>	2889 $\pm$ 492 <sup>1,2</sup> (n=17)	2830 $\pm$ 358 <sup>a,b,1</sup> (n=30)	2490 $\pm$ 377 <sup>2</sup> (n=24)

Values with the different numbered superscripts are significantly different within that group ( $p < 0.05$ ).

Values with different lettered superscripts in any column are significantly different at that time period ( $p < 0.05$ ).



**Figure 4.1 Transketolase Activity.**  
(Values with different letters are significantly different)

**Table 4.9 Thiamin pyrophosphate effect (TPPe) (% increase in transketolase activity)( $\bar{x} \pm \text{SD}$ ).**

	<b>Baseline</b>	<b>3 months</b>	<b>6 months</b>
<b>BM</b>	1.96 $\pm$ 4.2 (n=34) (1 > 15%)	7.03 $\pm$ 8.6 (n=31) (3 > 15%) (2 > 25%)	3.82 $\pm$ 4.6 (n=25)
<b>F</b>	1.74 $\pm$ 5.4 (n=31) (1 > 25%)	5.15 $\pm$ 6.7 (n=30) (4 > 15%)	2.70 $\pm$ 3.5 (n=26)
<b>EM</b>	2.67 $\pm$ 5.4 (n=19) (1 > 15%)	6.55 $\pm$ 9.8 (n=26) (2 > 15%) (1 > 25%)	7.08 $\pm$ 11.3 (n=23) (1 > 15%) (2 > 25%)

There were no differences between any groups at any time period.

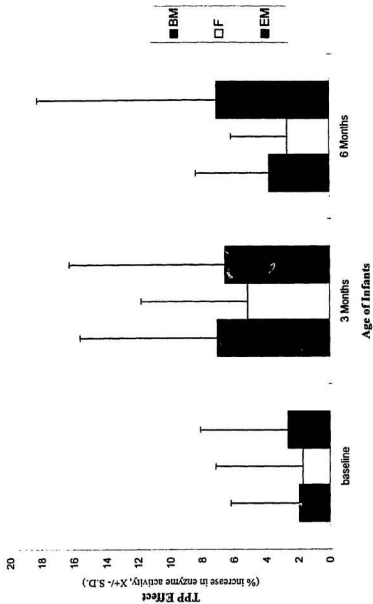


Figure 4.2 Thiamin Pyrophosphate Effect.

**Table 4.10 Fatty acid composition (wt %) of breastmilk (3 and 6 mos samples), formula, and evaporated milk ( $\bar{x} \pm SD$ ).**

<b>Fatty Acid</b>	<b>BM 3 mos (n=29)</b>	<b>BM 6 mos (n=10)</b>	<b>Enfalac® (n=3)</b>	<b>Similac® (n=5)</b>	<b>EM (n=6)</b>
<b>8:0</b>	0.07±0.06	0.11±0.04	0.98±0.04	1.48±0.17	0.55±0.13
<b>10:0</b>	1.14±0.37	1.14±0.26	1.26±0.19	2.15±0.08	2.29±0.28
<b>12:0</b>	5.45±1.61	4.93±1.32	10.7±1.66	19.67±0.58	3.38±0.11
<b>14:0</b>	6.16±1.53	5.71±1.86	4.92±0.50	8.33±0.23	11.79±0.35
<b>14:1</b>	0.25±0.09	0.24±0.06	ND	ND	1.01±0.07
<b>16:0</b>	17.35±1.90	17.91±1.46	21.82±0.34	10.42±0.16	31.25±1.77
<b>16:1(n-7)</b>	3.00±0.82	2.89±0.66	0.15±0.01	0.09±0.00	1.80±0.04
<b>18:0</b>	6.6±1.13	6.84±0.80	5.67±0.34	4.91±0.13	11.51±0.50
<b>18:1</b>	39.8 ±3.28	38.54±4.03	33.69±0.77	15.75±0.35	23.33±1.57
<b>18:2(n-6)</b>	12.11±2.91	12.61±1.50	17.1±1.41	30.47±0.51	2.27±0.32
<b>18:3(n-3)</b>	1.16±0.38	1.26±0.31	1.80±0.15	4.91±0.20	0.78±0.07
<b>20:0</b>	0.20±0.08	0.21±0.04	ND	ND	ND
<b>20:1</b>	0.64±0.20	0.48±0.08	ND	ND	0.03±0.03
<b>20:2(n-6)</b>	0.23±0.04	0.23±0.05	ND	ND	0.03±0.04
<b>20:3(n-6)</b>	0.29±0.07	0.28±0.07	ND	ND	0.09±0.01
<b>20:4(n-6)</b>	0.38±0.10	0.42±0.11	ND	ND	0.15±0.01
<b>20:5(n-3)</b>	0.13±0.14	0.07±0.02	ND	ND	0.09±0.03
<b>22:0</b>	0.10±0.06	0.14±0.04	0.27±0.02	0.22±0.02	0.12±0.02
<b>22:4(n-6)</b>	0.07±0.04	0.08±0.02	ND	ND	0.03±0.04
<b>22:5(n-3)</b>	0.14±0.07	0.16±0.03	ND	ND	0.37±0.13
<b>22:5(n-6)</b>					
<b>24:0</b>	0.06±0.06	0.07±0.05	0.14±0.03	0.11±0.00	0.06±0.05
<b>22:6(n-3)</b>	0.21±0.21	0.17±0.06	ND	ND	ND
<b>24:1</b>	0.04±0.04	0.05±0.04	ND	ND	ND
<b>18:2/18:3</b>	10.4/1	10.0/1	9.5/1	6.2/1	2.9/1

Similac® (16%), primarily as oleic acid (18:1). The essential fatty acids linoleic and  $\alpha$ -linolenic, are the highest in Similac® and the lowest in evaporated milk. The long chain polyunsaturated fatty acids (LCPUFA) of the n-3 and n-6 series, are not present in the commercial formulas. Breastmilk contained about 1.5% LCPUFA of which 0.4 % was arachidonic acid (AA, 20:4(n-6)) and 0.2% was docosahexaenoic acid (DHA, 22:6(n-3)). EM contained 0.15% 20:4(n-6) and no 22:6(n-3). There were no differences in breastmilk fatty acid composition between the three and six month samples.

#### **4.5.2 Erythrocyte phospholipid**

The results of the erythrocyte PE fatty acid analyses are presented in table 4.12. There were no significant differences at baseline. The predominant fatty acids were palmitic (16:0), stearic (18:0), oleic (18:1), arachidonic (20:4(n-6)), adrenic (22:4(n-6)), and docosahexaenoic acids (22:6(n-3)).

##### **4.5.2.1 Saturates**

Palmitic (16:0) and stearic (18:0) were the predominant saturated fatty acids at about 20% and 15% respectively. The concentration of 16:0 was significantly lower in breastfed infants at three, but not at six months, than the other two groups with a significant decrease from baseline to three months for all groups. There was no significant differences by feed group for the other saturated fatty acids, 18:0, 22:0, or 24:0. There were, however, some significant changes with time, as the concentration rose with age for 22:0 and 24:0.

**Table 4.11 Erythrocyte PE fatty acid composition (wt%,  $\pm$ S.D.)**

Fatty acid	Baseline			3 months			6 months		
	BM (n=22)	F (n=25)	EM (n=19)	BM (n=28)	F (n=27)	EM (n=25)	BM (n=17)	F (n=22)	EM (n=22)
14:0	1.0 $\pm$ 0.7	0.8 $\pm$ 0.7	0.7 $\pm$ 0.4 <sup>1</sup>	0.7 $\pm$ 0.2	0.8 $\pm$ 0.4	0.8 $\pm$ 0.4 <sup>1,2</sup>	1.2 $\pm$ 0.7	1.2 $\pm$ 0.5	1.1 $\pm$ 0.6 <sup>2</sup>
16:0	26.0 $\pm$ 2.8 <sup>1</sup>	26.4 $\pm$ 4.4 <sup>1</sup>	26.7 $\pm$ 3.5 <sup>1</sup>	19.7 $\pm$ 2.7 <sup>2,x</sup>	21.4 $\pm$ 3.1 <sup>2,y</sup>	21.9 $\pm$ 3.0 <sup>2,y</sup>	19.9 $\pm$ 2.7 <sup>2</sup>	19.7 $\pm$ 3.0 <sup>2</sup>	21.6 $\pm$ 3.8 <sup>2</sup>
16:1(n-7)	1.4 $\pm$ 1.1 <sup>1,x</sup>	1.1 $\pm$ 0.8 <sup>1,x</sup>	0.6 $\pm$ 0.6 <sup>1,y</sup>	0.9 $\pm$ 0.8 <sup>1,x</sup>	0.4 $\pm$ 0.6 <sup>2,y</sup>	1.9 $\pm$ 0.6 <sup>2,x</sup>	0.7 $\pm$ 0.6 <sup>1,y</sup>	0.2 $\pm$ 0.3 <sup>2,x</sup>	0.2 $\pm$ 0.6 <sup>2,x</sup>
18:0	17.4 $\pm$ 6.1	16.0 $\pm$ 6.1	14.1 $\pm$ 6.2 <sup>1,2</sup>	16.2 $\pm$ 3.9	14.9 $\pm$ 2.7	16.6 $\pm$ 5.9 <sup>1</sup>	13.8 $\pm$ 3.4	15.2 $\pm$ 6.8	12.9 $\pm$ 2.4 <sup>2</sup>
18:1(n-9+n-7)	15.4 $\pm$ 2.6 <sup>1</sup>	16.3 $\pm$ 2.7 <sup>1</sup>	17.0 $\pm$ 2.2 <sup>1</sup>	22.4 $\pm$ 2.3 <sup>2,x</sup>	19.7 $\pm$ 1.7 <sup>2,y</sup>	20.7 $\pm$ 2.3 <sup>2,x</sup>	21.4 $\pm$ 2.1 <sup>2,x</sup>	18.1 $\pm$ 2.6 <sup>1,y</sup>	20.7 $\pm$ 2.5 <sup>2,x</sup>
18:2(n-6)	2.6 $\pm$ 1.1 <sup>1</sup>	2.6 $\pm$ 0.7 <sup>1</sup>	2.9 $\pm$ 0.9 <sup>1</sup>	5.0 $\pm$ 1.2 <sup>2,x</sup>	10.8 $\pm$ 1.6 <sup>2,y</sup>	3.5 $\pm$ 0.8 <sup>1,x</sup>	7.3 $\pm$ 2.5 <sup>1,x</sup>	12.2 $\pm$ 2.5 <sup>2,y</sup>	5.4 $\pm$ 1.3 <sup>2,x</sup>
18:3(n-3)	ND	ND <sup>1</sup>	ND <sup>1</sup>	0.02 $\pm$ 0.1 <sup>x</sup>	0.2 $\pm$ 0.2 <sup>2,y</sup>	0.1 $\pm$ 0.1 <sup>1,2,x</sup>	0.1 $\pm$ 0.2	0.2 $\pm$ 0.3 <sup>2</sup>	0.2 $\pm$ 0.2 <sup>2</sup>
20:1	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3 <sup>1</sup>	0.2 $\pm$ 0.3	0.2 $\pm$ 0.3	0.3 $\pm$ 0.3 <sup>2</sup>	0.2 $\pm$ 0.3	0.4 $\pm$ 0.4	0.3 $\pm$ 0.4 <sup>2</sup>	0.3 $\pm$ 0.3
20:2(n-6)	0.2 $\pm$ 0.3	0.1 $\pm$ 0.1 <sup>1</sup>	ND	0.1 $\pm$ 0.2 <sup>x</sup>	0.9 $\pm$ 0.7 <sup>2,y</sup>	0.5 $\pm$ 0.6 <sup>x</sup>	0.1 $\pm$ 0.3	0.5 $\pm$ 0.6 <sup>2</sup>	0.1 $\pm$ 0.3
20:3(n-6)	1.5 $\pm$ 0.8	1.8 $\pm$ 0.8	1.4 $\pm$ 0.8	1.0 $\pm$ 0.7 <sup>x</sup>	1.5 $\pm$ 0.6 <sup>y</sup>	1.5 $\pm$ 0.6 <sup>y</sup>	1.0 $\pm$ 0.6 <sup>x</sup>	1.6 $\pm$ 0.6 <sup>y</sup>	1.4 $\pm$ 0.4 <sup>y</sup>
20:3(n-9)	0.02 $\pm$ 0.1	0.03 $\pm$ 0.1	0.03 $\pm$ 0.1	0.01 $\pm$ 0.04	0.04 $\pm$ 0.1	0.03 $\pm$ 0.1	0.03 $\pm$ 0.1	0.03 $\pm$ 0.1	0.2 $\pm$ 0.5
20:4(n-6)	18.4 $\pm$ 3.4	20.3 $\pm$ 3.8 <sup>1</sup>	21.1 $\pm$ 3.9 <sup>1</sup>	20.3 $\pm$ 2.8 <sup>x</sup>	18.3 $\pm$ 2.7 <sup>1,2,y</sup>	18.7 $\pm$ 2.7 <sup>1,2,y</sup>	18.8 $\pm$ 2.5	17.4 $\pm$ 2.6 <sup>2</sup>	17.4 $\pm$ 3.4 <sup>2</sup>

Fatty acid	EM (n=22)	Baseline F (n=25)	EM (n=19)	BM (n=28)	3 months F (n=27)	EM (n=25)	BM (n=17)	6 months F (n=22)	EM (n=22)
20:5(n-3)	0.2± 0.7	0.2± 0.5 <sup>1</sup>	0.1± 0.2 <sup>1</sup>	0.4±0.8 <sup>x</sup>	0.1±0.2 <sup>1,x</sup>	1.9±0.7 <sup>2,y</sup>	0.9±0.9 <sup>x</sup>	0.7± 0.7 <sup>2,x</sup>	2.7± 1.4 <sup>2,y</sup>
22:0	ND <sup>1</sup>	ND <sup>1</sup>	ND <sup>1</sup>	0.01 ±0.04 <sup>1</sup>	0.01 ±0.1 <sup>1,2</sup>	0.1 ±0.2 <sup>1,2</sup>	0.5 ±0.4 <sup>2</sup>	0.3 ±0.5 <sup>2</sup>	0.4 ±0.5 <sup>2</sup>
22:4(n-6)	6.6± 1.6	7.0± 1.5 <sup>1</sup>	6.7± 1.2 <sup>1</sup>	5.6±1.3 <sup>x</sup>	5.7±1.3 <sup>1,2,x</sup>	3.9±0.8 <sup>2,y</sup>	5.5± 1.1 <sup>x</sup>	5.8 ±1.6 <sup>2,x</sup>	4.1± 1.1 <sup>2,y</sup>
22:5(n-3+n-6)	0.3 ±0.4 <sup>1</sup>	0.3± 0.5 <sup>1</sup>	0.2± 0.3 <sup>1</sup>	1.7±0.9 <sup>2,x</sup>	1.6±0.8 <sup>1,2,x</sup>	3.3±1.2 <sup>2,y</sup>	2.3 ±1.4 <sup>2,x</sup>	2.4± 0.9 <sup>2,x</sup>	4.7± 1.4 <sup>3,y</sup>
24:0	0.1±0.4	0.2±0.6	0.2±0.8 <sup>1,2</sup>	0.03 ±0.1	0.2 ±0.4	0.1 ±0.4 <sup>1</sup>	0.6 ±0.7	0.7 ±1.0	0.7 ±0.9 <sup>2</sup>
22:6(n-3)	8.0± 2.4 <sup>1</sup>	7.6± 1.9 <sup>1</sup>	7.9± 1.9 <sup>1</sup>	6.0±1.7 <sup>2,x</sup>	3.1±0.8 <sup>2,y</sup>	4.1±0.9 <sup>2,x</sup>	5.3± 1.6 <sup>3,x</sup>	2.9± 0.8 <sup>2,y</sup>	4.2± 1.3 <sup>2,x</sup>
24:1	0.2±0.5	0.2±0.4	0.2±0.6 <sup>1</sup>	0.1 ±0.5	0.02 ±0.1	0.1 ±0.4 <sup>1</sup>	1.0 ±0.7	0.7 ±0.8	1.0 ±0.8

<sup>1,2,3</sup> Values with different numbered superscripts are different within groups over time (p<.05).

<sup>x,y,z</sup> Values with different lettered superscripts are different between groups at that time period (p<.05).



#### **4.5.2.2 Monounsaturated fatty acids**

With the exception of oleic acid, the monounsaturated fatty acids were found in very low concentrations. The EM fed group had significantly higher palmitoleic acid (16:1) at three months, followed by BM group, then F group. However, at six months the BM group was higher than both other groups. The concentration of 18:1 was higher in BM and EM groups than the F group at three and six months and at three months BM was higher than EM as well. It increased from baseline to three months of age for all groups. The concentration of 20:1 was very low and did not change with time. There was no difference in 24:1 between groups, but it increased between three and six months.

#### **4.5.2.3 N-6 fatty acids**

The concentrations of the major n-6 fatty acids are illustrated in figure 4.3. The concentration of linoleic acid correlated with milk fatty acid composition at both three and six months ( $r = 0.90$ , three months,  $r = 0.82$ , six months,  $p < 0.01$ ), and all groups were significantly different. In all groups 18:2(n-6) concentration increased with time. The concentration of 20:4(n-6) was significantly higher in the BM group than the other two groups at three months, however this difference disappeared at 6 months. Both F and EM groups were lower at 6 months than baseline, however there was no effect of time in breastfed infants. The concentration of 20:3 (n-6), was significantly lower in the BM fed than F and EM fed infants and did not change with time. The EM group had a significantly lower concentration of 22:4(n-6) at three and six months than BM and F groups. BM levels of 22:4(n-6) did not change with time, however, EM decreased from

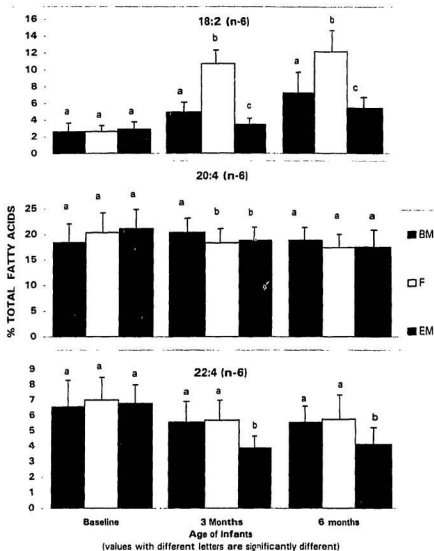


Figure 4.3 N-6 Fatty Acids.

baseline to three months and F decreased from baseline to six months.

#### **4.5.2.4 N-3 fatty acids**

The results for the predominant n-3 fatty acids are illustrated in figure 4.4.  $\alpha$ -Linolenic acid was present in very small amounts (<0.2%). The F group was slightly higher at three months than the other two groups. The concentration of eicosapentanoic acid (EPA, 20:5 (n-3)) was higher in the EM group than both F and BM infants, and increased with time in all groups. The concentration of 22:6(n-3) was significantly higher in the BM group, followed by EM fed infants, with F fed infants having the lowest concentration at both three and six months. Its concentration decreased from baseline to 3 months for all groups where it stabilized. The concentration of 22:5 was significantly higher in the EM group, than either the F or BM groups and it increased significantly with time for all groups. The n-6 and n-3 isomers were not resolved for 22:5, however based on previous research (Clarke et al, 1992, Sanders et al, 1979) 22:5(n-3) has been found greater at lower n-6/n-3 ratios than formula fed (higher 18:2 /18:3(n-3)) or breastfed infants with no change in the n-6 isomer, demonstrating that the predominant isomer in EM fed infants is the n-3.

#### **4.6 Visual acuity**

Breastfed infants had significantly higher visual acuity than EM fed infants at both three and six months of age and F fed infants were not different from either group (table 4.12, figure 4.5). All values were within normal range for healthy full term infants at three and six months of age (Courage et al, 1990). Visual acuity did not correlate with

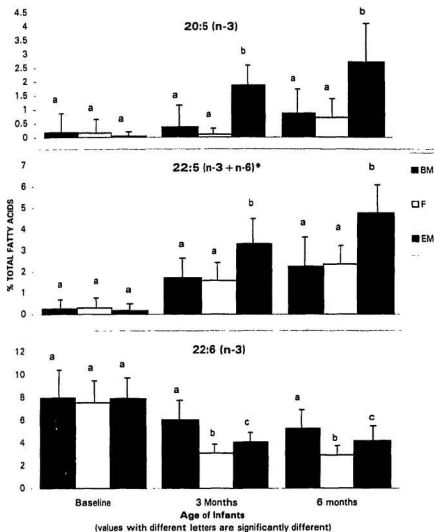


Figure 4.4 N-3 Fatty Acids.

either erythrocyte PE 22:6(n-3) or dietary intakes of 22:6(n-3) when tested for the entire group of infants or within feeding groups. The frequency distribution of subjects at each acuity card score (cycles/degree) is illustrated in figure 4.6. The higher numbers of EM fed infants at the lower end, as well as the higher numbers of BM fed infants at the upper end of the scale, illustrates the differences between groups.

**Table 4.12 Visual acuity (cycles/degree,  $\pm$  SD).**

	<b>BM</b>	<b>F</b>	<b>EM</b>
<b>3 Months</b>	3.86 $\pm$ 0.29 <sup>a</sup> (n=34)	3.46 $\pm$ 0.34 <sup>ab</sup> (n=33)	3.29 $\pm$ 0.41 <sup>b</sup> (n=29)
<b>6 months</b>	9.03 $\pm$ 0.29 <sup>a</sup> (n=29)	8.41 $\pm$ 0.35 <sup>ab</sup> (n=30)	7.54 $\pm$ 0.25 <sup>b</sup> (n=30)

Values with different superscripts in any row are significantly different at that time period ( $p < 0.05$ ).

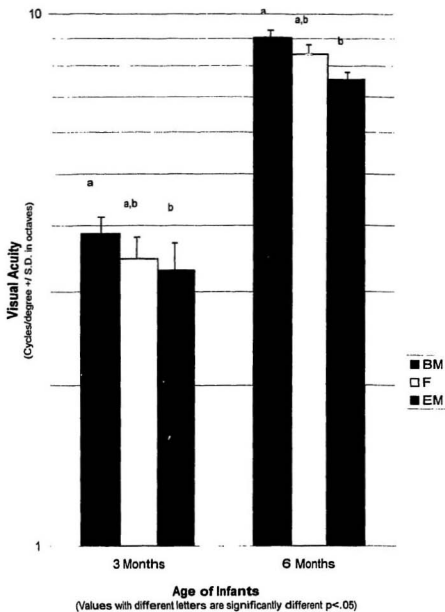
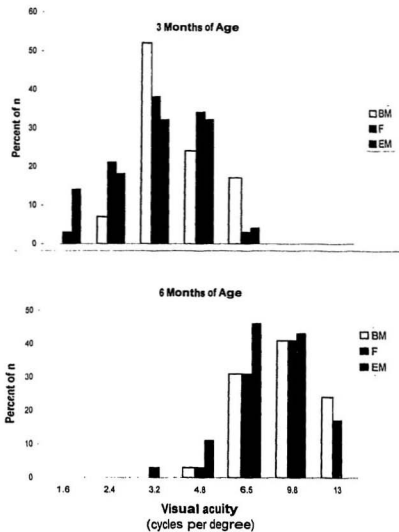


Figure 4.5. Visual Acuity.



**Figure 4.6. Frequency Distribution of Visual Acuity Scores.**



### 5.1 Growth

The question of what constitutes "normal" growth for infants has remained a problem in the construction of percentile curves. The US National Centre for Health Statistics (NCHS) is currently the most widely used standard reference values. Most of these infants were either exclusively bottle fed or breastfed for a short period of time (Hamill et al, 1977). Key changes in infant feeding have occurred since this data was obtained. The later introduction of solid foods, the humanization of infant formulas, and the increase in breastfeeding, have all contributed to a different reference group (Dewey, et al, 1992). The humanization of infant formulas would have involved the conversion from evaporated milk formula to present day formulas.

Overall, the infants in the present study were on average larger than the NCHS reference values, from birth to 6 months. The infants had high birth weights, at about the 75th percentile, with average birth lengths. Using  $z$  scores, which determine the standard deviation from the median of NCHS standards, all groups had higher weights and lengths at three and six months of age. There was, however, an apparent "faltering" in the breastfed group in weight gain between three and six months. This is shown in weight gain velocity, head circumference increase velocity, and  $z$  scores for weight for age and weight for length. Although the breastfed infants remain at or above reference median values, their rate of growth is reduced compared to non-breastfed infants.

The larger infants in the present study are in agreement with the DARLING study

(Davis Area Research on Lactation, Infant Nutrition and Growth, Dewey et al, 1992), as well as Duncan et al (1984) demonstrating that there may be a secular trend towards larger infants since the NCHS data was gathered. In the DARLING study, until 6-7 months of age, the infants in all age groups had greater length and weight than the NCHS medians regardless of type of feeding. In that study, the investigators actually corrected for the high birth weight in their population before determining z-scores. Subjects in that study were of high education and socioeconomic status. In contrast, mothers of infants in the present study were of a broad range of education and socioeconomic status. However, the weight and lengths of breastfed and non breastfed infants at all comparable ages were very similar to the present study, demonstrating that socioeconomic status is not a factor. In Duncan et al (1984), infants were also larger than the NCHS medians, starting at the 60-70th percentiles, and even with a decrease in growth velocity, breastfed infants only drop to the 50th percentile by 6 months of age. This provides another important reason, besides differences in growth between breastfed and formula fed infants, for revising the current growth charts in order to reflect advances in pre- and postnatal care as well as the general shift in recent years to a taller population.

Most studies have shown differences in weight gain between three and six months, however, differences in length between formula and breastfed infants are conflicting. Duncan et al (1984), found that the majority of breastfed infants did not follow the NCHS curves, losing an average of 20 percentiles in weight for age and 30 percentiles in length. Chandra (1982), found differences in weight from the NCHS percentiles but no differences in length. Czaka-Narins et al (1986) and the DARLING

study found similar results when comparing breastfed with bottle fed infants. In the DARLING study the two cohorts were matched for parental socioeconomic status, ethnic group, education, and for infant sex and birth weight, and with no introduction of solid foods for the first four months. They found that the mean weight of breastfed infants was significantly lower than the formula fed groups between 6 and 18 months of age. However, there were no differences in length or head circumference or length gain velocity. These results suggest that breastfed infants are leaner and not necessarily smaller than those formula fed. Czajka-Narins et al (1986) reported similar results. They found that linear growth of breastfed infants was no different than a comparison group of formula fed infants. Breastfed males were lighter than those fed formula at 6 and 12 months of age, with no significant differences at 24 months of age. Using weight for length indices fewer infants who were breastfed were categorized as being overweight.

In the present study, linear growth or linear growth velocity did not differ, however, in agreement with previous studies, weight gain between three and six months of age was reduced compared to both other formula groups. This is also seen in the  $z$  scores which show a decline in weight for length and weight for age from three months to six months of age in breastfed but not in the other formula groups. However, in contrast to previous studies, there was a slight but significant difference in head circumference growth velocity between 3 and 6 months of age.

The growth of evaporated milk and formula fed infants was almost identical. This was expected since numerous studies since the first use of evaporated milk in infant feeding in the 1920's have shown consistently that evaporated milk fed babies grew as

well or better than breastfed infants (Apple, 1987). In the first major study of evaporated milk, 1422 infants were studied between 1927 and 1929. They found that evaporated milk fed infants regained their birthweight quicker than those fed a modified cow's milk formula or exclusively breastfed. The average weight gain was similar for all groups (Marriott et al, 1929). The similar growth of formula fed and EM fed infants demonstrates that the humanization of formulas has not had any effect on growth.

## **5.2 Thiamin**

Thiamin deficiency in infants, which expresses itself as infantile beri-beri, is rare. Its instances have been confined to the breastfed infants of thiamin deficient mothers in Asian countries where polished rice is the staple food (Thanangkul et al, 1966). In North America it has been limited to infants fed a soy based formula, which at the time contained almost no thiamin (Davis et al, 1958, Cochrane et al., 1961). There is very little information on the thiamin needs of infants, resulting in requirements which are estimated from the requirements of adults. In establishing requirements, urinary thiamin excretion, transketolase activity, and the TPP effect are often used as biochemical indicators of thiamin status. Excretion correlates with thiamin intake to a critical point after which further lowering in the range of 0.2-0.4 mg/1000 kcal, results in only variable and minor changes in excretion. Therefore, as intakes decrease below 0.3 mg/ 1000kcal, urinary thiamin excretion starts to plateau, and the TPPe rises above 15% indicating a functional deficiency of thiamin. (Saubertlich, 1981). These results form the basis of the requirement for all age groups of 0.4 mg/1000 kcal (Nutrition Recommendations, 1990),

which has a safety factor included.

In this study, at three months, 94% of BM fed, 68% of EM fed and no F fed infants have intakes which fall below this recommendation. With supplementation, weaning and solid food introduction at six months this drops to 21% of EM fed and 28% of BM fed below the RNI. However, deficiencies in breastfed infants of well nourished mothers or EM fed have not been reported.

The extrapolation of requirements for infants based on adults may not be appropriate. Studies show that results of biochemical indices of thiamin status in infants differ from older age groups. Infants maintain an increased level of transketolase activity (TKA) up to the first year of life compared to young children and adolescents, and reach the level of adulthood at about the age of ten years. As well, the TKA in cord blood is higher than in maternal blood (Markkanen et al, 1971). This finding agrees with the observation that fetomaternal transfer is positive on the side of the fetus (Tripathy, 1968). Changbumrung et al (1984) found, in a study of 518 infants and small children, a markedly higher transketolase activity in children than in their mothers, but no differences between infants within the ages of 0-60 months. The urinary excretion of thiamin, expressed on a creatine basis also is much higher in children than adults (Stearns et al, 1958). More recently, whole blood thiamin and thiamin in cerebrospinal fluid was determined in infants up to one year of age. Both indices decreased over the first twelve and eighteen months of life respectively and then stabilized thereafter (Wyatt et al, 1991). In the present study, a change with age was also seen. All feeding groups showed a drop in transketolase activity between three and six months of age, however only the breastfed

infants had an increase in the TPP effect between birth and three months. These changes make it clear that thiamin requirements of infants have to be determined separately from other age groups. It is important to determine age specific requirements for infants because they may be most at risk for long term effects of thiamin deficiency because such a deficiency has neurological consequences (Haas, 1988).

There is only one known study of comparing thiamin status of infants on different feedings. In this study, the excretion of thiamin in the feces of infants was higher in breastfed infants than those on formula or a mixture of formula and breastmilk (Kusaka, 1968). Information on thiamin intakes were not given, but at the time, the formula was probably similar to EM. This result may indicate that either there was poorer thiamin absorption in breastfed infants, or breastfed infant had better intake and were thus excreting more thiamin.

In the present study, the transketolase activity and the TPPe were measured as an indication of thiamin status. This assay provides information on tissue reserves of thiamin and reflects its functional adequacy. There were no differences in the TPP effect between groups, and the combined values match those of other studies. The average range of 2-7% in this study is similar to that found in infants and young children in Bangkok (1-60 months of age, TPPe = 9%) (Changbumgrung et al, 1984), and in Germany (<1 year of age, TPPe = 8%) (Reinken et al, 1979).

The percent of infants in the marginal or deficient range for TPPe in the present study are similar to Reinken et al (1979) in which 8.5% and 1.1% of infants aged up to year were marginal and deficient respectively. At three months, 10% of the breastfed

infants were marginally deficient and 7% were deficient, with none marginal or deficient at six months of age. The evaporated milk fed group with similar intakes had 8% marginal, and 4% deficient at three months of age. However, at six months there remained 4% marginal and 9% deficient in the EM group. None of the formula fed infants were deficient in thiamin at any time and 13% were marginally deficient at three months and none were at 6 months. This appears to indicate that EM fed infants may be at greater risk for developing thiamin deficiency. However, an inadequate intake of thiamin does not appear to be responsible for the marginal and deficient values, since only one of the infants with marginal or deficient values had an intake below the RNI (0.19 mg/1000kcal). These results indicate that complete tissue saturation of thiamin may not be required for normal health, indicating that a wide range for the TPP effect may be acceptable, without indicating a deficiency of thiamin.

The lower enzyme activity of breastfed infants at three months of age but without a correspondingly high TPP effect may indicate that breastfed infants may already be saturated in thiamin but have a lower maximal transketolase activity. This is demonstrated by the correlation seen at three months between energy intake and transketolase activity ( $r = 0.28$ ,  $p = 0.003$ ) but not with TPP effect or thiamin intake. The possibility exists that at the lower energy intakes as seen in breastfed infants less transketolase production is induced. Sauberlich (1979) demonstrated the interrelationship between energy intake and thiamin requirement in adults, in which men consuming 2800 kcal required less thiamin than those consuming 3600 kcal to maintain adequate status according to the TPPe and urinary thiamin excretion. At six months of age, as infants are

weaned and solid foods are being introduced, the energy intake increases, and the correlation disappears. The lower transketolase activity appears not to be responsible for the lower weight gain between three and six months of age as there was no linear correlation either overall or within feeding groups.

### **5.3 Fatty acids**

#### **5.3.1 Milk composition**

##### **5.3.1.1 Human milk**

The fatty acid composition of the breastmilk in the present study is similar to the milk of other Canadian, Australian, and American mothers on mixed diets (Makrides et al, 1995, Putnam et al, 1982, Innis et al, 1994). Breastmilk fatty acid composition can vary greatly, depending on the diet. In particular, the concentration of the essential fatty acids, linoleic and  $\alpha$ -linolenic acid, is dependent on the amount and type of vegetable oil in the mother's diet. As such, a vegetarian diet will produce higher milk 18:2(n-6) levels than an omnivorous diet. However, although the subject variability is quite high the means from study to study of differing geographic location is quite similar. Thus the mean linoleic acid composition is 11% in a combination of several studies of European mothers, 12% in African mothers (Koletzko et al, 1992), 13.5% in both British Columbian (Innis et al, 1994), and Australian mothers (Makrides et al, 1995), however the ranges were wide, from 5% to 22%. As well, a mean value of 29% from vegetarian



women has been determined (Innis et al, 1988). The mean value found in the current study of 12.1% at three months and 12.6% at 6 months is in agreement with women on mixed omnivorous diets.

The content of the n-6 LCPUFA, however, is much less responsive to maternal diet, and a relatively constant value of 0.4-0.5% for 20:4(n-6), the primary n-6 LCPUFA, is seen (Koletzko et al, 1992, Makrides et al, 1995, Innis et al, 1994). This occurs even in vegetarian women whose diets are low in animal fat containing virtually no n-6 LCPUFA (Koletzko et al, 1992, Innis et al, 1988). The concentration of 22:6(n-3) is somewhat responsive to maternal diet with greater amounts in the milk of mother's consuming a diet high in marine or animal fat (Innis, 1992). Ranges are between 0.1-0.9%, with most in the 0.2-0.3% range as in the current study, with a value as high as 1.9% in marine-oil supplemented women (Harris et al, 1984). Although 22:6(n-3) can vary greatly, it is still the primary n-3 LCPUFA in breastmilk even in mothers consuming high amounts of 20:5(n-3). This maintenance of 22:6(n-3) and 20:4(n-6) in human milk demonstrates that, besides diet, metabolic processes may also be a factor in regulating the amount of LCPUFA in human milk.

#### **5.3.1.2 Commercial formula**

The manufacture of commercial formulas involves skimming cow's milk, and adding vegetable oil blends in varying proportions to get the desired final fatty acid composition. The two commercial formulas used in the study, Enfalac® and Similac®, are quite different in their composition. Enfalac® contains a soya and corn oil blend, whearas

Similac® contains corn, coconut and soya oil blend (Label information, 1994). Enfalac®, as a result, is very similar to human milk in the amount of saturated, monounsaturated, and essential fatty acids. Similac is less saturated but contains much higher amounts of the essential fatty acids. Neither formula contains LCPUFAs as these can only come from an animal source.

### **5.3.1.3 Evaporated milk**

Evaporated milk formulas are highly saturated and contain very low amounts of the essential fatty acids. Cow's milk fat is more complex in composition than is vegetable oil or human milk fat because it contains many positional and geometric isomers of the monounsaturated and polyunsaturated fatty acids, products of biohydrogenation in the rumen of the cow. Linoleic acid, in particular, besides the biologically active *cis-cis* 18:2(n-6) also has appreciable amounts of the *cis-trans* and *trans-trans* isomers which are not separated by conventional gas liquid chromatography. Therefore, only 50-80% of the 18:2(n-6) in butterfat is the active form (Sanders et al, 1979). Thus the actual value of 18:2(n-6) may only be 1.2-1.8% of total fatty acids, rather than the total value of 2.3% reported in this study. As well, Sanders et al, (1979) determined that 20:3(n-3) is not resolved from 20:4(n-6), and butterfat, unlike human milk fat, contains primarily 20:3(n-3) as well as some 20:4(n-3). Thus, the value of 0.15% of total fatty acids for 20:4(n-6) in EM reported in this study may be an overestimate. The amount of 22:5 (n-3) (0.37%) is much higher than that previously found (0.10%) (Sanders et al, 1979). Whether this is due to variations in cow milk supply or there were other unresolved fatty acids included

in this peak is unknown.

### **5.3.2 Clinical deficiency signs**

There were no obvious clinical signs of essential fatty acid deficiency in any of the study infants in any group. These signs include dry scaliness or thickening of the skin and reduced growth. This is expected since this condition has only been seen in infants fed 0.07 and 0.04 % kcal as linoleic acid (Hansen et al, 1963), patients on fat free TPN (Paulsrud et al, 1972), rats fed either fat free (Burr et al, 1929) or <0.14% of energy as 18:2(n-6) (Holman, 1960). Using the method of a "dermal score" Mohrhauer et al, (1963) found that in rats, 0.6% of energy as linoleic acid or 0.25% as 20:4(n-6) prevented deficiency symptoms. Infants or rats fed diets based on butterfat, similar to evaporated milk, containing less than 1% energy as linoleic acid have never been known to exhibit signs of clinical EFA deficiency (Hansen 1963, Naismith et al, 1978, Holman, 1960, Holman et al, 1965).

### **5.3.3 Blood**

#### **5.3.3.1 Circulating fatty acid composition is a function of both fatty acid ratios and absolute intakes**

As fatty acids of the n-3, n-6, and n-9 series compete for the same enzymes in the preferential order n-3>n-6>n-9, the balance in the dietary intake between each series as well as their absolute amounts of preformed LCPUFAs will determine the relative rates of

production of the LCPUFA's. This knowledge allows predictions of the effect of feeding various fatty acid blends on tissue or circulating fatty acid composition. In the present study, the high dietary intake of linoleic acid and absence of LCPUFA in the vegetable oil based formulas would result in higher concentrations of circulating linoleic acid and lower amounts of n-3 LCPUFAs. The evaporated milk fed group, as a reflection of a low dietary n-6/n-3 ratio due to the preferential desaturation of 18:3 (n-3), would produce relatively more n-3 products than the other groups. As well, as both n-6 and n-3 fatty acids are in low amounts, some production of the n-9 metabolites, particularly 20:3(n-9) may occur. The composition of certain LCPUFAs, notably 20:4(n-6) and 22:6(n-3), in the circulation of breastfed infants would not be as easy to predict, as breastmilk contains preformed sources. These are very bioactive and appear to be directly incorporated into membranes, with little turnover or oxidization for energy (Innis, 1991).

#### **5.3.3.2 N-9 FATTY ACIDS**

A classical biochemical marker of essential fatty acid deficiency is the triene /tetraene ratio. Limited intakes of both essential fatty acids allows the accumulation of trienes, primarily eicosatrienoic acid (20:3 (n-9)) along with a reduction in tetraene (20:4(n-6)). N-9 fatty acids are formed from oleic acid, which can be produced either from carbohydrate de novo or obtained from the diet. Curves relating linoleic acid intake to the triene/tetraene ratio have been constructed for various organs in rats which have shown a sharp rise in triene accompanied by the reduction in tetraene, (20:4 (n-6)) at intakes of linoleic acid below 1% of energy (Mohrauer et al, 1963).

Elevated triene values as compared to breastfed infants have been found when a butterfat based milk has been fed to infants in plasma (Naismith et al, 1978, Holman, 1965, Hanson et al, 1963) and erythrocytes (Sanders et al, 1979, Clark et al, 1992).

In the present study, however, only trace amounts of 20:3(n-9) were found and there were no differences between groups. There was, however, higher concentrations of 18:1(n-9) in EM fed than those F fed, which is also associated with a decline in available essential fatty acids (Sanders et al, 1979). The level was not as high as breastfed infants due to their high intake of 18:1(n-9). The erythrocyte PE fraction has a slower turnover than either plasma or total erythrocyte lipid and is therefore more resistant to dietary changes. Therefore, as in other studies, although there may have been a rise in plasma or total erythrocyte lipid n-9 levels, it was unlikely at these intakes to have risen in erythrocyte PE or any organs, especially the brain. Also low zinc and copper intake is known to impair  $\Delta 6$  desaturase activity thus reducing the formation of 20:3(n-9) and 20:4(n-6) (Cunnane et al, 1995, Cunnane, 1985). As EM is known to be low in both zinc and copper, 20:3 (n-9) production may have been impaired.

In studies to date, there has been no measurement of organ accretion of 20:3 (n-9) in piglets or human infants fed limited 18:2(n-6). However, rat studies have shown a slight increase over a large range of essential fatty acids intakes in brain, liver and heart, with the brain the most resistant to incorporation (Mohrauer et al, 1963). These studies, however, were done in the early days of GC analysis, and as there was no n-6 triene value reported, the n-6 and n-9 isomers of 20:3(n-9) may not have been resolved. In a more recent study, there was no 20:3 (n-9) found in any organs in rats fed a minimum of 0.3%

energy as 18:2(n-6) and 0.3% energy as 18:3(n-3) (Bourre et al., 1990).

### **5.3.3.3 N-6 fatty acids**

#### **5.3.3.3.1 Linoleic acid (18:2(n-6))**

The amount of 18:2(n-6) incorporation correlated linearly with the percent of total fatty acids in the diet at three and six months. In rats, this linear trend with intake occurs for most organs outside of the CNS, however the CNS, including the retina, nerve endings, myelin, and brain with the exception of the sciatic nerve, contains only about 1% LA, which does not change with intake (Bourre et al, 1990).

However, within the BM group, there was no linear trend between the concentration of 18:2(n-6) in the breastmilk and its circulating PE concentration. This is supported in piglet studies in which sow milk containing either 8 or 23% of circulating fatty acids as 18:2(n-6) produced no differences in circulating 18:2(n-6) (Innis, 1993). In contrast, formulas containing varying amounts of 18:2(n-6) have corresponding differences in circulation (Ponder et al, 1992, Putnam et al, 1982). This has been explained by a difference in the metabolism of 18:2(n-6) between breast and formula fed infants. The reduced plasma LDL and cholesterol in formula fed infants as a reflection of reduced cholesterol and saturated fat intakes (Jensen, 1989) may produce a delay in 18:2(n-6) turnover, resulting in an accumulation of 18:2(n-6) in plasma and subsequently red cell phospholipids in the circulation of formula fed infants (Innis, 1993).

#### 5.3.3.3.2 Arachidonic acid (20:4(n-6))

At three months of age, breastfed infants had higher 20:4 (n-6) concentrations than formula or EM fed infants, but for different reasons. The difference is partly due to the amount of preformed 20:4 in breastmilk. However, in formula fed infants, the known inhibition of  $\Delta$  5 desaturase (20:3 (n-6)  $\rightarrow$  20:4 (n-6)) by high 18:2(n-6) intakes could have reduced its production (Brenner et al, 1969). Differences in 20:4(n-6) between breast and formula fed infants are commonly seen in plasma and total red cell lipids. However in erythrocyte PE, results are conflicting, depending on the duration of the study, the ratio of 18:2(n-6)/18:3(n-3), and the absolute amount of 18:2(n-6) in the diet (Innis, 1991). The levels seen in EM fed infants are simply due to low 18:2(n-6) intakes as previously reported by Sanders et al, (1979) and Clarke et al, (1992).

Despite differences between formula fed and breastfed circulating levels of 20:4(n-6), in the CNS these levels are tightly controlled, indicating that the selective uptake of 20:4(n-6) may allow sufficient accretion in the organs that require it (Makrides, 1994). In rats, only 0.3% of energy as linoleic acid was required for the brain to accrete stable amounts of 20:4(n-6). However, the liver required 2.4% of energy as 18:2(n-6) (Bourre, et al, 1990). Using this evidence, although circulating levels may match those in the F group, EM fed infants with <1% energy as 18:2(n-6) may not be accreting an optimal level of 20:4(n-6) in certain organs outside the CNS, however, there may be adequate accretion within. The differences seen between BM and F groups in 20:4(n-6), probably as a result of high formula 18:2(n-6) intakes, may not extend to organ accretion. In both piglets and human infants, formula fed infants have similar accretion of 20:4(n-6)

as those fed human or sow milk, although circulating levels may differ (Makrides et al, 1994, Hrboticky et al, 1990, Arbuckle et al, 1992).

#### **5.3.3.3.3 Adrenic acid (22:4(n-6))**

The low intake of 18:2 (n-6) in the EM group produced a significant reduction in the concentration of 22:4(n-6) as compared to formula or breastfed infants as shown by a linear correlation with intake ( $r = 0.48$ , three months,  $r = 0.45$ , 6 months,  $p < 0.004$ ). This is supported by other studies in which low intakes of 18:2(n-6) (<1.7% energy) reduce organ (Bourre et al, 1990) and circulating concentrations (Naismith et al, 1979, Clarke et al, 1993) of 22:4(n-6).

Breastfed and formula fed infants will differ in erythrocyte PE concentrations of 22:4(n-6) only when there is a high ratio of n-6/n-3 (>9:1) fatty acids in the formula (Ponder et al, 1992, Innis et al, 1994). This is in agreement with the present study in which the majority of infants in the F group were fed a formula with a ratio of 6:1 (18:2(n-6)/18:3(n-3)), produced similar circulating levels of PE 22:4(n-6) to those seen in the BM group.

The specific role of 22:4 (n-6) in organs has not been elucidated, however, it comprises a significant portion of brain lipids, about 7% of the cerebral cortex in human infants (Farquharson et al, 1992, Makrides et al, 1994). In the rat brain, when 18:3(n-3) intakes are adequate, 2.4 % of energy as 18:2(n-6) is required to support maximal 22:4(n-6) accretion (Bourre et al, 1990). However, at inadequate 18:3(n-3) intakes autopsy results on infants and piglet studies have demonstrated a compensatory rise in 22:4(n-6)



and 22:5(n-6) (Hrboticky et al, 1990, Farquharson et al, 1992, Makrides et al, 1994).

#### **5.3.3.4 N-3 fatty acids**

##### **5.3.3.4.1 $\alpha$ -Linolenic acid (18:3(n-3))**

18:3 (n-3), the primary precursor to fatty acids of the n-3 series, is known to be present in extremely low amounts in both the circulation and in organs. In this study, in contrast to the accumulation of 18:2(n-6), there was < 0.3% 18:3(n-3) at all time periods in all feeding groups and was undetected in many subjects.  $\alpha$ -Linolenic acid, in comparison to the other 18 carbon fatty acids, is quickly desaturated and elongated to its bioactive products, and unlike 18:2(n-6) does not seem to support a major structural role in membranes. As well, it is more easily oxidized for energy as it has a greater affinity for the acylcoenzyme A transport system than 18:2(n-6) (Innis, 1992).

##### **5.3.3.4.2 Eicosapentaenoic acid (20:5(n-3))**

With the exception of the EM group, 20:5(n-3) was present in erythrocyte PE in very low amounts. (<1%). This is also seen in other studies of breastfed and formula fed infants in both organs and in circulation (Makrides et al, 1994, Farquharson et al, 1992, Ponder et al 1992, Innis et al, 1994).

In contrast to the other two groups, the EM fed infants had an accumulation of 20:5(n-3) in erythrocyte PE. An explanation may be the favourable n-3/n-6 ratio in evaporated milk, but it also appears to be the direct result of low 18:2(n-6) intakes. Low

18:2(n-6) intakes associated with a sharp rise in 20:5(n-3) has been seen in circulating levels in animals (McMurchie, 1990) and infants (Clarke, 1993) and in the liver, lung, and kidney of the rat (Bourre, 1990). A partial explanation is the compensatory replacement of 20:4(n-6) with 20:5(n-3) from the competition at the  $\Delta 5$  desaturase position. In McMurchie et al, however, when marmoset monkeys were supplemented with 20:5(n-3), the increase in erythrocyte phospholipids in 20:5(n-3) was mirrored by a decline in 18:2(n-6) and not 20:4(n-6). They then hypothesized that 18:2(n-6) and 20:5(n-3) occupy a similar spot in membranes, which 20:5(n-3) will occupy during limiting 18:2(n-6) intakes (McMurchie et al, 1990). This is supported by the results from Clarke et al (1992) in which fullterm infants fed formulas with similar ratios of n-3/n-6 (3-4:1) but different absolute amounts of 18:2(n-6) (6.4% vs 1.7%) and 18:3(n-3) (1.7% vs 0.5%) produced similar 22:5(n-3) and 22:6(n-3) in erythrocytes. However, the formula with lower 18:2(n-6) produced significantly higher 20:5(n-3) in circulation. This supports the possibility of a unique structural role for 18:2(n-6) itself and not just its desaturated and elongated products. Therefore, an increased incorporation of 20:5(n-3) may be a marker of limited 18:2(n-6) intakes in infants. Although an elevated level of 20:5(n-3) may be beneficial in the prevention of thrombosis and heart disease in an adult population (Sinclair, 1992) it may reduce eicosanoid formation in developing infants.

#### **5.3.3.4.3 DOCOSAPENTAENOIC ACID (22:5 (n-3+n-6))**

Evaporated milk fed infants had higher docosapentenoic acid (22:5(n-3+n-6)) values than formula or breastfed infants. Although the isomers were not resolved in the

present study, previous research suggests that the difference is due to the elevation in the n-3 isomer as a result of a low n-6/n-3 ratio. Previously, Sanders et al (1979), found that evaporated milk fed infants had significantly higher concentrations of erythrocyte 22:5 (n-3) but equal 22:5 (n-6) to breastfed infants. This is explained by the low n-6/n-3 ratio, which is favourable to n-3 production, and does not seem to be related to the absolute amounts of 18:3(n-3) in the diet. Low 18:2(n-6) intake may allow more  $\Delta 6$  and  $\Delta 5$  desaturation of 18:3(n-3), or alternately, low 18:2(n-6) intake may downregulate peroxisomal retroconversion to 22:6(n-3). This is supported by Clarke et al (1992), who found that even at 3-fold amounts of both 18:2(n-6) and 18:3(n-3), if given in similar ratios of 3 or 4:1 still have similar levels of 22:5 (n-3) and 22:5 (n-6) levels in erythrocyte lipids. At this low a ratio, concentrations of 22:5(n-3) were also higher than in breastfed infants.

At the higher dietary 18:2(n-6)/18:3(n-3) ratios (6:1-9:1) of other studies, similar to the commercial formulas used in the present study, 22:5 (n-3) concentrations are lower in formula than breastfed infants, and 22:5 (n-6) levels are either higher or the same at three months of age (Innis et al, 1994, Ponder et al, 1992). Thus the equal amounts seen here of 22:5 in the breast and formula fed infants suggests that the n-3 isomer makes up a larger proportion of 22:5 in breastfed infants, with the reverse being so for the n-6 isomer.

22:5 (n-3) is not one of the major fatty acids in circulation or in organs (Makrides et al, 1994, Martinez, 1992). However, increased 22:5 (n-6) incorporation in tissue lipids to compensate for reduced 22:6 (n-3) is a characteristic feature of n-3 deficiency. In infants fed formula, autopsy analyses demonstrated significantly higher 22:5 (n-6)

accretion in the cerebral cortex, although circulating levels did not reflect it (Farquharson et al 1992, Makrides et al, 1994). Blood lipid 22:5 (n-6) is rarely different between breast and formula fed infants or piglets (Innis, 1992), therefore the relative n-3 to n-6 levels would probably be very similar in this study. In rat organs, a level of 18:3 (n-3) of 0.4% energy produced a plateauing of both 22:6(n-3) and 22:5(n-6), after which 22:5 (n-6) did not decrease further, and inversely 22:6(n-3) did not increase further (Bourre et al, 1989).

#### **5.3.3.4.4 Docosahexaenoic acid (22:6(n-3))**

As seen so far, the very low ratio of n-6/n-3 in EM, due to the lack of n-6 competition, produces more n-3 LCPUFA in circulation than formula or breastfed infants. This occurred for all n-3 fatty acids but 22:6(n-3). 22:6(n-3) was significantly higher at both three and six months in the breastfed than in EM or formula fed infants. This is a finding commonly seen in erythrocyte phospholipids of term infants (Ponder et al, 1992, Putnam et al, 1982, Innis, 1991). The presence of preformed 22:6(n-3) in the human milk provides an explanation.

The reason for greater accumulation of 22:5 (n-3) in EM fed than breastfed infants, but with lower 22:6(n-3) production provides evidence that the conversion of 22:5(n-3) to 22:6(n-3) is slow in infants. This would indicate that a preformed source of is a more efficient supplier than precursors of 22:6(n-3). It has been known for some time that reaction rates at the  $\Delta 6$  and  $\Delta 5$  positions are about the same, however at the  $\Delta 4$  position (22:5(n-3) to 22:6(n-3) ) rates are much slower (Bernet et al, 1975). In explanation, it was recently discovered that there is no  $\Delta 4$  desaturase enzyme but a several

step conversion. This conversion comprises of an elongation step, a  $\Delta 6$  desaturation, and subsequent  $\beta$ -oxidation (Sprecher, 1992). The complexity of this conversion would imply that substrate specificity and competitive interactions between substrates affect the rate of this step.

The 22:6(n-3) content in erythrocyte PE was in the order BM>EM>F as a reflection of the competitive nature of the of the  $\Delta 6$  desaturase, as well as the preformed 22:6(n-3) in human milk. However, evidence, primarily in piglets, has shown that circulating levels are not always a reflection of the amount incorporated into organs, particularly the CNS.

Evidence for this is most dramatically seen in studies of piglets. Piglets fed either sow milk or marine oil based formula with a preformed source of 22:6(n-3) have higher circulating 22:6(n-3) than piglets fed a vegetable oil based formula containing no 22:6(n-3). The difference was related to the amount of preformed 22:6(n-3) in the diet and it occurred whether the levels in the brain, liver, or retina were normal or reduced (Arbuckle et al, 1992, Hrboticky, 1990). However, when no preformed source of 22:6(n-3) is provided in the diet, it is the ratio of 18:2(n-6)/18:3(n-3) that determines the levels in circulation. This occurred in piglets, in which two formulas which had similar n-6/n-3 ratios of 16/4 or 10/2, and no 22:6(n-3) produced the same erythrocyte circulating levels of 22:6(n-3). However, the brain and liver had significantly greater 22:6(n-3) in animals fed 1.5% 18:3(n-3) rather than 0.75% 18:3(n-3) (Innis, 1992). Therefore, the concentrations of circulating fatty acids may be deceptive in providing information concerning the adequacy of the fatty acids in the diet. Therefore, animal studies, autopsy

analyses of infant tissue, and functional testing, must also be used to determine the adequacy of the diet.

i) *Animal Studies.* In rats, 0.3% kcal for the whole brain and 0.7% kcal for the synaptic membranes and retina phospholipids as 18:3(n-3) was required to produce a maximal accretion of 22:6(n-3). In piglets, 0.8% (0.4% kcal) as  $\alpha$ -linolenic acid does not produce levels seen in those fed sow milk fed, however, 2% kcal as 18:3(n-3) does (Hrboticky et al, 1990, Arbuckle et al, 1992). Again in piglets, the brain and liver accumulated significantly more 22:6(n-3) at a 18:3(n-3) intake of 1.5% kcal than at 0.75% kcal (Innis, 1992).

ii) *Autopsy results.* Autopsy results of term infants have shown that formula fed infants have both lower circulating (Makrides et al, 1994) and cerebral cortex incorporation (Farquharson et al, 1992, Makrides et al, 1994) of 22:6(n-3), but with no difference in the retina (Makrides et al, 1994) than those breastfed. The amounts of 18:3(n-3) in these formulas, however, were low. The formula fed infants in the study by Makrides et al, consumed diets containing only 1-1.6% fatty acids (0.5-0.8% kcal) as 18:3(n-3). In the study by Farquharson, all formula diets contained < 0.75% kcal as 18:3(n-3).

iii) *Visual function.* As direct analyses of CNS 22:6(n-3) accretion in relation to dietary n-3 intake is not possible in healthy infants, functional tests relating to intake and circulating fatty acid levels must be used. Decreased 22:6(n-3) in the retina and brain of rodents and non human primates fed diets deficient in 18:3 (n-3) has been shown to be accompanied by altered learning behaviours, electroretinogram recordings, and decreased

visual acuity (Innis, 1991). In this study, visual acuity was determined as a functional test of fatty acid adequacy.

The results from the present study of diets containing 1% or 2.5% of energy as 18:3(n-3) show that visual acuity is matched with breastfed infants, despite much lower concentrations of PE 22:6(n-3). However, evaporated milk fed infants on diets of only 0.3% of energy as 18:3(n-3) show significantly lower visual acuity at both three and six months than those breastfed.

Results from other studies have shown that levels of 0.4% (Uauy et al, 1992) or 0.5-0.8% (Makrides et al, 1994) of energy as 18:3(n-3) without a preformed source of 22:6(n-3), produced either lower visual acuity or visual evoked potential (VEP) than a comparison group of breastfed infants. Makrides et al (1995), studied infants fed a LCPUFA supplemented formula, an unsupplemented formula with 0.8% energy as 18:3(n-3), or breastfed. Those without the LCPUFA supplementation had significantly lower VEP than the other groups. However, Innis, et al (1994), found no difference in visual acuity in healthy term 3 month old infants breastfed or fed formula with 17% 18:2(n-6) and 2% 18:3(n-3) of total fatty acids (1% energy as 18:3(n-3)). However, it must be noted that the method of visual testing differed. VEP is considered to have better acuity thresholds, and matures more rapidly than behaviour (PL) methods used in the present study and in the study by Innis et al (1994) (Lampkin, 1992).

From these results, it appears that in the absence of preformed 22:6(n-3), 1% kcal may be the minimum requirement of 18:3(n-3) for optimum visual function for infants. Many formulas, as well as EM, do not meet this requirement. The commercial formulas

used in this study Similac® (Ross laboratories) and Enfalac® (Mead Johnson) are most common in Canada, and appear to be adequate. In summary, for full term infants, 22:6(n-3) in the diet may not be required to produce optimum visual function provided that there is adequate 18:3(n-3) in the formula.

Although the intake of 18:3(n-3) appears to be a predictor of visual acuity, circulating 22:6(n-3) levels is not. There was not a correlation between circulating 22:6(n-3) and visual acuity. Evaporated milk fed infants had circulating 22:6(n-3) levels higher than those formula fed. However, formula fed infants demonstrated no difference when compared to either group in visual acuity. This result agrees with that obtained from piglet studies, mentioned above, in which although the ratios of n-6/n-3 produce differences in erythrocyte lipids, it was the absolute amount of 18:3(n-3) that determined the amount of 22:6(n-3) accretion in organs. In contrast, Makrides et al, (1994) found a correlation between circulating 22:6(n-3) and visually evoked potential in breastfed and formula fed infants. However, higher 22:6(n-3) in circulation may have been due to a preformed source, and the low dietary 18:3(n-3) in the formula supported inadequate retinal accretion of 22:6(n-3), expressing itself as reduced visual evoked potential.

Using the results of the above studies the requirement for n-3 fatty acids can be determined to be about 1% of energy as 18:3(n-3) in the absence of a dietary supply of 22:6(n-3). In rats, piglets, and infants, diets containing 0.7-0.8% of energy as 18:3(n-3) did not support adequate accretion of 22:6(n-3). Functional results using visual testing have shown that term infants require 1% of energy as 18:3(n-3) for optimal visual development. Therefore, the evaporated milk fed infants in this study with intakes of



only 0.3% of energy as 18:3(n-3) were likely deficient in 18:3(n-3).

## **CHAPTER 6.0      CONCLUSIONS**

### **Subjects**

- In Newfoundland, the chosen method of infant feeding is related to age, education and socioeconomic status. Mothers feeding evaporated milk tend to be younger, less educated, and of lower socioeconomic status. Those breastfeeding are more educated, and of higher socioeconomic status, while those formula feeding are in the intermediate range. Therefore, education targeting the less advantaged about breastfeeding benefits appears to be required.

### **Growth**

- The infants in the present study were larger for their age than standard reference values (NCHS), with the exception of breastfed infants at six months of age who matched the reference. A larger sample size is required to determine whether there is a secular change to larger infants since the NCHS data was obtained.
- Breastfed infants demonstrated the characteristic lag in weight gain velocity behind bottle fed infants between three and six months of age. However, length, and length gain velocity was equal for all groups. The reference values may need to be revised to accommodate the differing growth rates of breastfed infants. In contrast to previous studies, breastfed infants had lower head circumference growth velocity between three and six months of age. The significance of this finding is unknown.
- Infants fed either evaporated milk or commercial formulas had similar rates of

growth.

### **Thiamin**

- There is no difference in the thiamin pyrophosphate effect between infant feeding groups at any time period. However, transketolase activity in breastfed infants is lower than formula fed infants at three months of age. It correlated with energy, but not thiamin intake, indicating that the lower energy intake in breastfed infants may be the cause and not a lack of thiamin.
- All groups had some infants in the marginal or deficient range for TPP effect at three months of age, and breastmilk and evaporated milk fed groups had some infants in this range at 6 months of age. Other research on healthy infants (Reinken et al, 1979) has provided similar results. Whether the criteria determined by Brin et al (1965), is too strict in determining adequacy of thiamin intakes is unknown, but from the present results a wide range of TPP effect appears to be compatible with health. Further study using other biochemical indices of thiamin status such as the urinary excretion of thiamin would provide more answers.

### **Fatty acids**

- Evaporated milk fed infants have, with the exception of 20:4(n-6), lower n-6 fatty acids in circulation than formula fed or breastfed infants, due to lower intakes. 20:4(n-6) was lower in EM and F fed infants than breastfed infants at three but not six months of age. Whether this difference is reflected in various organs with possible functional consequences is unknown. Further research in animals of the

effects of varying levels of dietary n-6 fatty acids on organ accretion of n-6 LCPUFA's is needed to determine requirements.

- With the exception of 22:6(n-3) in breastfed infants, EM fed infants have higher n-3 fatty acids than the other groups. However, this result is misleading as animal research has shown that circulating n-3 fatty acids are not a good index of organ accretion of LCPUFA's. The concentration of 22:6(n-3) in circulation appears to be a reflection of the amount of preformed source in the diet, as well as dietary n-6/n-3 ratios and is not necessarily reflected in organs, particularly those of the CNS.
- The low  $\alpha$ -linolenic acid intakes (<0.3% kcal) in the evaporated milk fed group may have led to decreased visual acuity, as compared to breastfed infants. There is much support in the literature to indicate that this level is inadequate to support optimal organ accretion of n-3 fatty acids and optimal visual development. In this study, the adequacy of the  $\alpha$ -linolenic acid amount in commercial formulas cannot be ascertained as the visual acuity in this group did not differ from the others. However, based on other research in animals and infants, the dietary level of 18:3(n-3) in the commercial formulas is adequate.

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## APPENDIX A

### MEMORIAL UNIVERSITY OF NEWFOUNDLAND ST. JOHN'S, NEWFOUNDLAND

#### CONSENT TO PARTICIPATE IN BIO-MEDICAL RESEARCH

TITLE. MEMORIAL UNIVERSITY INFANT FEEDING STUDY

INVESTIGATOR(S): JAMES K. FRIEL, WAYNE L. ANDREWS, DAVID R. LONG,  
ALLISON McDONALD, URSULA McCLOY

You have been asked to participate in a research study. Participation in this study is entirely voluntary. You may decide not to participate or may withdraw from the study at any time without affecting your normal treatment.

Confidentiality of information concerning participants will be maintained by the investigator. The investigator will be available during the study at all times should you have any problems or questions about the study.

The purpose of this study is to examine the effect of different feeding practices on the nutritional health of the infant. This information will help us in providing advice on the best way to feed infants. All infants will be followed in their chosen feedings.

We ask that at three and six months of age you come to a special clinic with your infant. At that time and before your baby leaves hospital a small sample of blood (less than a teaspoon) will be taken and weight and height measured. We also ask that you fill in prior to this clinic visit, a dietary questionnaire that will tell us what your child is eating.

The study will not have any risks to your infant. In order to reimburse your expenses and as acknowledgement for your participation in this study, we will provide \$40 per visit. At the end of the study we will give you all of the results.

If you choose not to enter the study, or wish to withdraw at any time, this will have no effect on the care you will receive.

I, \_\_\_\_\_, the undersigned, agree to my \_\_\_\_\_ participation in the research study described above.

Any questions have been answered and I understand what is involved in the study. I realize that participation is voluntary and that there is no guarantee that I will benefit from my involvement. I acknowledge that a copy of this form has been offered to me.

\_\_\_\_\_  
Signature of Participant

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Witness (Optional)

.....  
To be signed by Investigator:

To the best of my ability I have fully explained to the subject the nature of this research study. I have invited questions and provided answers. I believe that the subject fully understands the implications and voluntary nature of the study.

\_\_\_\_\_  
Signature of Investigator

\_\_\_\_\_  
Date

Telephone Number: \_\_\_\_\_



**APPENDIX B      Subject Information**

**INITIAL SCREENING - INFANT FEEDING STUDY**

**Name:** \_\_\_\_\_

**Residence:** \_\_\_\_\_

**Telephone #:** \_\_\_\_\_

**Age of Baby (Date of Birth):** \_\_\_\_\_

**Gestational Age:** \_\_\_\_\_

**Birth Weight:** \_\_\_\_\_

**Sex of Infant:** \_\_\_\_\_

**Were there any difficulties with the infant at birth or is there any at present?**  
**(congenital defects, heart, kidney, lung dysfunction, general illnesses)**

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**What are you feeding your infant at present?**

**Breastmilk** \_\_\_\_\_  
**Formula/ bottle** \_\_\_\_\_  
**(eg. Similac, Enfalac, etc.)**  
**Evaporated Milk** \_\_\_\_\_  
**(eg. Carnation, Pacific)**

**If breastfeeding, do you have any allergies?**  
**yes** \_\_\_\_\_ **no** \_\_\_\_\_

**Do you expect to continue with this type of feeding?**  
**yes** \_\_\_\_\_ **no** \_\_\_\_\_

**If not, to which type do you expect to change, and when?**

---

**Are you interested in participating in this study?**

yes \_\_\_\_ no \_\_\_\_

**FEEDING STUDY QUESTIONNAIRE**

DATE: \_\_\_\_\_ SUBJECT #: \_\_\_\_\_

INFANT NAME: \_\_\_\_\_ SEX: \_\_\_\_\_

GESTATIONAL AGE: \_\_\_\_\_ BIRTH WEIGHT: \_\_\_\_\_

MOTHER'S HEIGHT: \_\_\_\_\_ WEIGHT (prepregnancy): \_\_\_\_\_

MOTHER'S AGE: \_\_\_\_\_

MARITAL STATUS: Married \_\_\_\_\_ Single \_\_\_\_\_ Other \_\_\_\_\_

FATHER'S HEIGHT: \_\_\_\_\_

NUMBER OF CHILDREN: \_\_\_\_\_

MOTHER'S OCCUPATION: \_\_\_\_\_

FATHER'S OCCUPATION: \_\_\_\_\_

EDUCATION LEVEL OF MOTHER: \_\_\_\_\_  
(Highest grade level, postsecondary education, etc.)

Do you smoke? \_\_\_\_\_

If yes, # cigarettes per day: \_\_\_\_\_

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THE FOLLOWING QUESTIONS ARE TO BE COMPLETED EACH VISIT.

METHOD OF FEEDING: (check appropriate one)	IN HOSPITAL	VISIT 1	VISIT 2
Bottle/ formula (eg. Similac, Enfalac)	_____	_____	_____
Evaporated Milk Formula (eg. Carnation, Pacific)	_____	_____	_____

**Breast feeding**                    \_\_\_\_\_

**If formula feeding, how do you prepare your formula? (how much water do you add, if any, and do you add anything else to to your formula)**

**In hospital (how do you plan to prepare)** \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

**Visit 1 - three months (if different from above)** \_\_\_\_\_

\_\_\_\_\_

**Visit 2 - six months (if different from above)** \_\_\_\_\_

\_\_\_\_\_

**Will / do you consistently use the method of feeding indicated above? (eg. evaporated milk usually, ready made formula other times)**

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

**Do / will you give your infant vitamin or mineral supplementation?**

**YES**

**NO**

**In hospital**                    \_\_\_\_\_

**Visit 1**                                    \_\_\_\_\_

**Visit 2**                                    \_\_\_\_\_

**If yes check the appropriate one(s).**

	In hospital	Visit 1	Visit 2
Vitamin A & D	_____	_____	_____
Vitamin C	_____	_____	_____
Iron	_____	_____	_____
Fluoride	_____	_____	_____
other (please indicate)	_____ _____	_____ _____	_____ _____

Give details. (include quantity, form, when given, and brand name)

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**COLLECTION:****0 MONTHS   3 MONTHS   6 MONTHS****DATE****\_\_\_\_\_**

	<b>YES</b>	<b>NO</b>	<b>YES</b>	<b>NO</b>	<b>YES</b>	<b>NO</b>
<b>WEIGHT</b>	___	___	___	___	___	___
<b>HEAD CIRCUMFERENCE</b>	___	___	___	___	___	___
<b>LENGTH</b>	___	___	___	___	___	___
<b>DIET RECORD RECEIVED</b>	___	___	___	___	___	___
<b>BLOOD SAMPLE</b>	___	___	___	___	___	___
<b>PAYMENT MADE</b>	___	___	___	___	___	___
<b>METHOD OF FEEDING</b> (Yes if same as Previous)	___	___	___	___	___	___

**Indexing of occupation and education:**

**Occupation:** The socioeconomic index was determined using the Blishen scale, which takes into account both income and social status of the occupation in Canada (Blishen et al, 1976).

**Education:** An eight point scale was developed to grade levels of education obtained.

The scale was the following:

Elementary	1
Junior High	2
Part of High school	3
High School completed	4
Part of Trade School	5
Trade or Technical completed	6
Part of University degree	7
University degree completed or higher	8

## **APPENDIX C      Clinic Information for Parents**

Memorial University  
Infant Feeding Study

(Date)

Dear (name),

The three month visit of your infant to the infant feeding clinic is approaching. The following are several reminders:

1) We have enclosed a three day food record for you to fill out within the last week, and preferably the last three days before you come to the clinic. Since you have been requested to feed milk only for the first three months, the food record will be quite simple, indicating the amount, brand name, proportions used in mixing, and the time of each feeding. It will be reviewed with you when you come to the clinic.

2) If breastfeeding we ask that you hand express a sample of your milk and place it into the vial enclosed, either the morning of or the night before you come to the clinic, and bring it with you. Keep it stored in the fridge, but **please do not freeze it**. If feeding evaporated milk or formula, we will ask for a small sample (about a tablespoon) to be poured off from the bottle you are currently feeding your infant the day of the clinic, so please bring your baby's bottle with you.

3) The clinic will be held at the (hospital) on (date). At this time visual function and length and weight of your infant will be measured. A small blood sample will also be taken. We will call you a week before the clinic to provide details on the exact location and time of the appointment as well as to answer any questions you may have.

Thank you once again for your cooperation and participation in this study. We look forward to seeing you on (date). Your help will make life easier for all infants. For any questions or comments please call:

Claude Mercer, Ursula McCloy or  
Allison McDonald, R.N.  
737-8541  
Dr. James Friel,  
737-7954



Memo:ial University  
Infant Feeding Study

(Date)

Dear (name),

The six month visit of your infant to the infant feeding clinic is approaching. We are looking forward to seeing both of you again. The following are several reminders:

1) As before, we have enclosed a three day food record for you to fill out within the last week, and preferably the last three days before you come to the clinic. Remember to include any liquid or solid foods and supplements, indicating the amount, brand name, proportions used in mixing, and the time of each feeding. It will be reviewed with you when you come to the clinic.

2) If still breastfeeding we ask that you hand express a sample of your milk and place it into the vial enclosed, either the morning of or the night before you come to the clinic, and bring it with you. Keep it stored in the fridge, but **please do not freeze it**.

3) The clinic will be held at the (hospital), on (date). We will again measure visual function, head circumference and length and weight of your infant. A small blood sample will also be taken. We will call you shortly before the clinic to provide details on the exact location and time of the appointment as well as to answer any questions you may have. If you have not received a call, or if your number has changed, please call the university (737-8541) or Allison McDonald (747-2141) at home.

Thank you once again for your cooperation and participation in this study. We look forward to seeing you on (date). Your help will make life easier for all infants. For any questions or comments please call:

Claude Mercer, Ursula McCloy  
or Allison McDonald, R.N.  
737-8541  
Dr. James Friel,  
737-7954

## APPENDIX D

### Erythrocyte transketolase activity assay

#### Sample preparation

- 200 $\mu$ L of erythrocytes were diluted in equal amounts of distilled water.

#### Reagent preparation

- Buffer:

0.9% NaCl	20 mls
1.15% KCl	515 mls
1.75% $K_2HPO_4$	100 mls
3.82% $MgSO_4 \cdot 7H_2O$	5 mls

1N HCl was used to bring the pH to 7.4

- Thiamin Pyrophosphate (TPP)
  - a) Stock solution: 25 mg cocarboxylase (TPP): 25 ml buffer
  - b) Working solution: 1 ml stock solution: 16 ml buffer
- 5% Trichloroacetic acid
- Pentose standard solution
  - a) Stock solution: 1mg D-ribose: 1ml distilled water
  - b) Working solution: 1 ml stock solution: 100ml distilled water (10 $\mu$ g/ml)
- Orcinol reagent
  - a) 30% HCl: 3 parts concentrated HCl to one part distilled water
  - b) 

2 g orcinol	} to 50 mls water
0.1 g $FeCl_3 \cdot H_2O$	
  - c) dilute b) to 1000ml with 30% HCl
- Ribose-5- phosphate substrate: 0.084 g R-5-P disodium salt to 7 ml distilled water. Add buffer to 6.8 ml substrate to obtain a volume of 10 ml.

#### Calculations

Amount pentose utilized per ml hemolysate per hour:

- Dilution Factor (DF):  
 $DF = 1/0.1^a \times 3.6^b/1 \times 1/0.2^c = 180$   
^a amount of hemolysate

<sup>b</sup> total volume

<sup>c</sup> ml filtrate used

- Standard (SP): average absorbance (optical density, OD)/ $\mu$ g pentose standard  
 $SP = (OD_5/5 + OD_{10}/10) \div 2$
- Constant (KP) = DF/SP
- $2R + D$  = amount of pentose originally present in the group of tubes prior to incubation
- $TP_1 = (2R + D - A) \times KP = \mu$ g pentose used/ml hemolysate/hour without TPP
- $TP_2 = (2R + D - B) \times KP = \mu$ g pentose used/ml hemolysate/hour with TPP
- Thiamin pyrophosphate effect (TPPe) (%):  
 $TPPe = TP_2 - TP_1 / TP_1 \times 100$
- For contents of tubes A, B, D, and R refer to tables 3.1 and 3.2.  
A = absorbance without exogenous TPP  
B = absorbance with exogenous TPP  
D = absorbance of amount of pentose endogenous to the sample  
R = absorbance of original amount of substrate added
- Determination of basal transketolase activity (TKA):

To correct for between run variation, standard transketolase values of an adult control blood sample were obtained. The transketolase activity of this control was determined with every run of samples and a ratio of the standardized transketolase activity ( $TKA_s$ ) over the within run control transketolase activity ( $TKA_c$ ) was obtained. Thus, the corrected basal transketolase activity for each subject was calculated as:

$$TKA_{(corrected)} = (TKA_s / TKA_c) \div TP_1 \text{ (tka uncorrected)}$$





