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# Effects of Dietary (n-3) Fatty Acids on Erythrocytes and Other Haematological Parameters in Cynomolgus Monkeys (*Macaca fascicularis*)

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## Abstract

The relative effects of dietary (n-3) fatty acids from plant and fish were investigated in Cynomolgus monkeys. These sources provided fatty acids of different chain lengths which were tested under conditions to permit a constant intake of polyunsaturates and of (n-6) fatty acids. The erythrocyte was chosen as an index of (n-3) dietary history for the feeding period of fifteen weeks.

Dietary long chain (n-3) fatty acids, supplied with equal amounts of linoleate, had an impact on red cell fatty acid profiles. When a shorter chain (n-3), linolenate, from linseed oil was provided, also in equal amounts with linoleate, a constant ratio of total (n-6) to total (n-3) of 2.4 was maintained. The provision of longer chain (n-3) from fish (menhaden) oil, finally reduced this ratio to one-sixth, 0.39. Alterations in this ratio reflected those of the individual fatty acid components and reflected a close regulation of membrane acyl composition by the dietary source of fatty acids. The findings also demonstrated that a direct incorporation of preformed long chain (n-3) fatty acids, eicosapentaenoate and docosahexaenoate, circumvented cellular control which could have been exerted by desaturation/elongation. The (n-3) fatty acids must therefore be provided from a fish (menhaden) rather than a plant (linseed) source to obtain an elevated level of long chain (n-3) in the erythrocyte and to displace (n-6).

Female monkeys showed a greater response to the diet, by their relatively greater incorporation of (n-3) fatty acids and by their lower

incorporation of (n-6) fatty acids, as compared to males.

Two diets containing menhaden oil differed only in alpha-tocopherol contents. No diet-induced peroxidative stress was apparent from assays of erythrocyte superoxide dismutase, glutathione peroxidase and thiobarbituric acid.

Haematological properties (platelet cell counts, bleeding time and bleeding time blood volume) also showed no dietary effects, perhaps due to the equal provision of (n-6) with (n-3) fatty acids in the diets.

Serum cholesterol and triglycerides were not affected by these polyunsaturated diets.

## Dedication

This thesis is dedicated to my family, in appreciation for their constant support and encouragement and for their dedication to my education.

**Acknowledgements**

I would like to thank the many people who have made valuable contributions to the successful completion of this thesis.

I wish to thank those in the Nutrition Research Division, Bureau of Nutritional Sciences, Health and Welfare Canada, for sharing their laboratory space and equipment, providing helpful advice and continued encouragement. Special thanks to Rudy Hollywood for sharing his knowledge of gas chromatographic techniques, to Elaine O'Grady for her contribution to the cholesterol and triglyceride assays, to Svend Kaasgaard for his analysis of dietary tocopherol and to Nancy Versteeg for her assistance in the extractions and methylations. Gratefully acknowledged are the efforts of the Animal Resources Division, Health and Welfare Canada, for their diligent care of the animals.

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# Introduction

The essentiality of linoleic (18:2, n-6), the first member of the (n-6) family of fatty acids, was established by Burr and Burr in 1929; however, alpha-linolenic acid (18:3, n-3), the first member of the (n-3) series, has not unequivocally been shown to be essential (Marshall and Johnston, 1982). Neither linoleic (LA) nor linolenic (LNA) acids can be synthesized de novo in animal tissues, but both can undergo further proximal desaturation and elongation to form 20- and 22-carbon derivatives (refer to Figures A and B). Moreover, no interconversion occurs between the (n-6) and (n-3) series, as both compete for common enzymes for incorporation into the 2-position of glycerophospholipids of biomembranes. Linoleic acid, and its derivatives, gamma-linolenic (18:3), dihomo-gamma-linolenic (20:3), arachidonic (20:4), adrenic (22:4) and docosapentaenoic (22:5) are characteristic of vegetable or animal fats; while (n-3) fatty acids, eicosapentaenoic (20:5), docosapentaenoic (22:5) and docosahexaenoic (22:6) are obtained from seafood or derived from dietary 18:3 (n-3), which occurs in some seed oils such as canola, soybean and linseed and in green leafy vegetables.

## i: Dietary Manipulation of Fatty Acid Composition

The essentiality of the (n-3) family has been questioned since some, but not all, symptoms of essential fatty acid deficiency can be normalized by its intake. Recent studies have raised the possibility that (n-3) fatty acids, docosahexaenoic (DHA) in particular, may be essential as vital structural components of specialized membranes: retina, synaptosomes of cerebral cortex, spermatozoa and testes (Tinoco, Babcock, Hincenbergs, Medwadowski, Miljanich and Williams, 1979).

**Figure A:**

i) The 3 fatty acid families and their nomenclature

Fatty acid nomenclature uses trivial names, chemical names and shorthand notation of chemical structure, interchangeably. Shorthand notation provides the total number of carbon atoms:number of unsaturated double bonds. Unsaturated fatty acids are further divided into three main classes according to the position of the first double bond counting from the terminal methyl group (or omega, 'w' carbon) of the carbon chain; giving rise to the 'w-' or 'n-'3, 6 and 9 series. Refer to appendix.

Harris, Connor and McMurry (1983a)

ii) (n-6) family of fatty acids are substrates of the monoenoic and dienoic eicosanoids; (n-3) fatty acids are precursors of trienoic eicosanoids.

Hansen (1983)

## FATTY ACID NOMENCLATURE

## DIETARY SOURCES (MAJOR)

i)

| FAMILY | FATTY ACID | STRUCTURE | DIETARY SOURCES (MAJOR) |
|---|---|---|---|
| ω3 | Eicosopentaenoic Acid (C20:5 ω3) | $H_3C$ 3 RCOOH | Marine Oils, Fish |
| ω6 | Linoleic Acid (C18:2 ω6) | $H_3C$ 6 RCOOH | Vegetable Oils |
| ω9 | Oleic Acid (C18:1 ω9) | $H_3C$ 9 RCOOH | Vegetable Oils; Animal Fats |

ii)

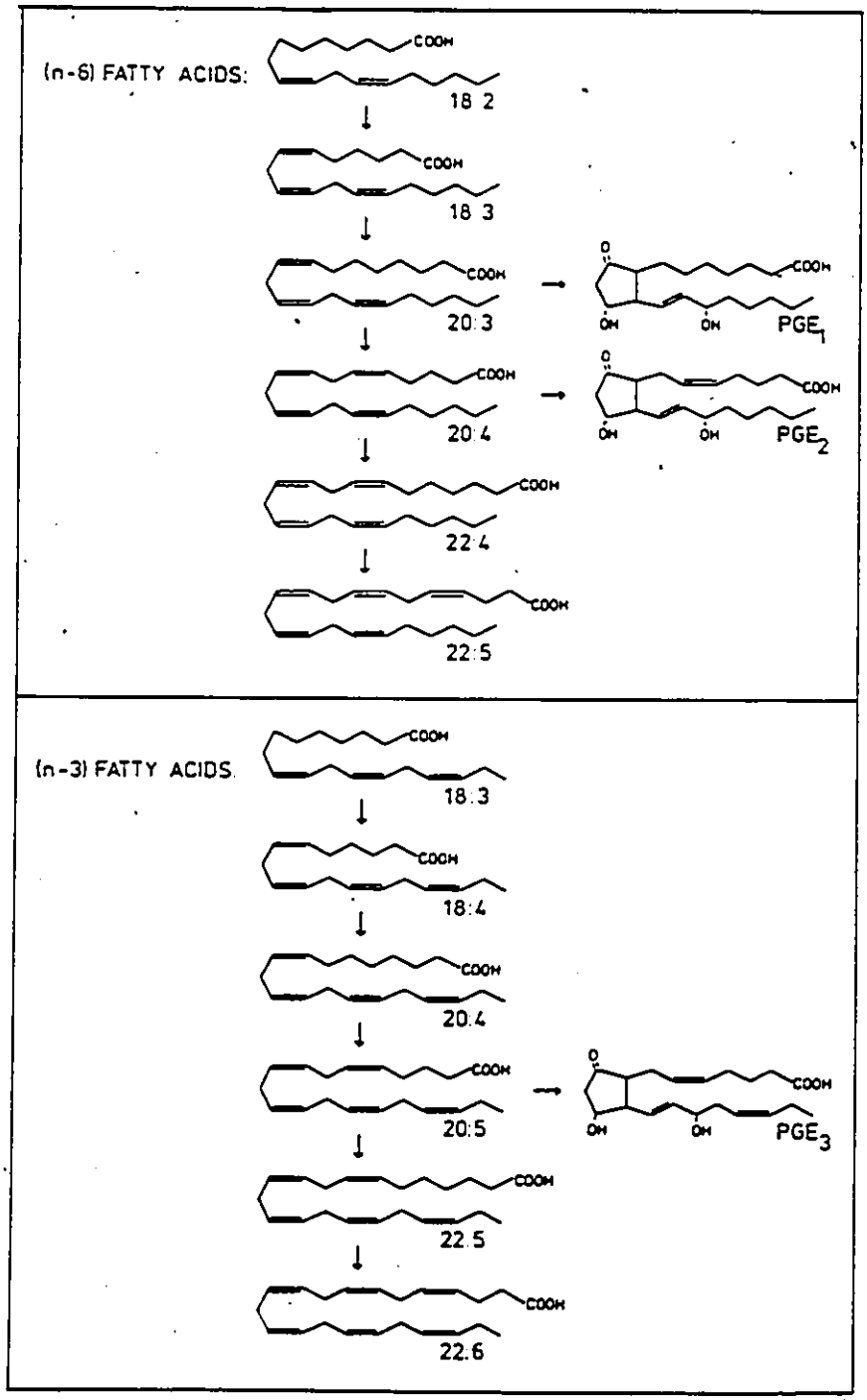
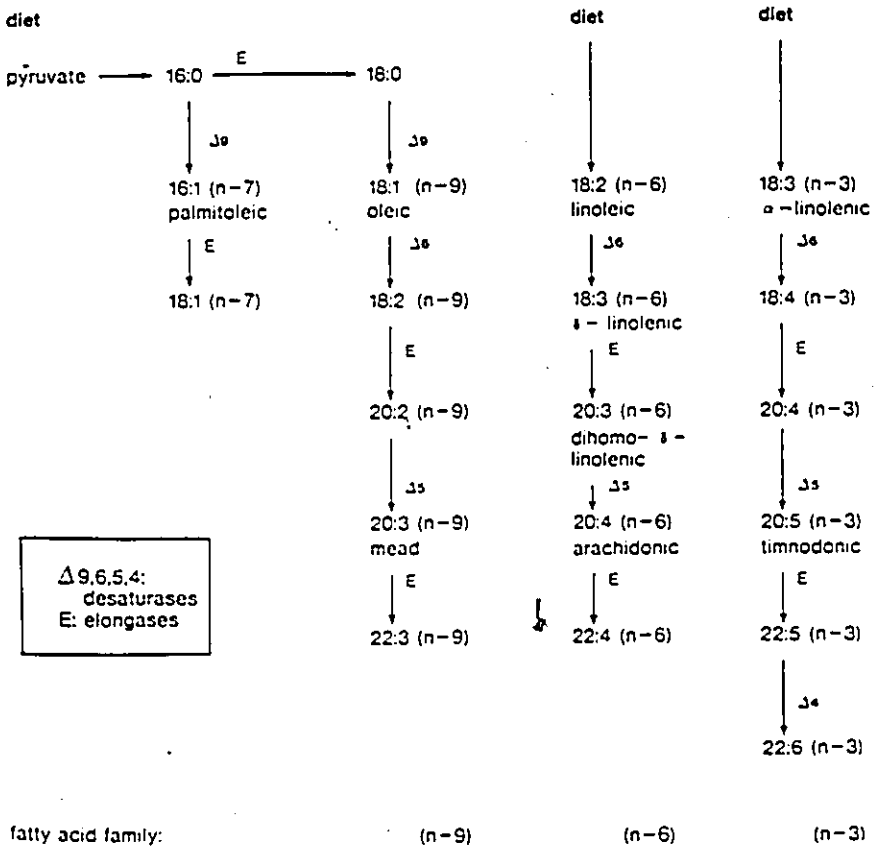


**Figure B:**

Metabolic pathways indicating elongation and desaturation of the three families of fatty acids: (n-9), (n-6) and (n-3).

Fatty acids are sequentially desaturated and elongated, at specific carbons by the desaturase and elongase enzymes in the liver and in some tissues.

Hornstra (1982)


diet
pyruvate
16:0
E
18:0
Δ9
16:1 (n−7)
palmitoleic
E
18:1 (n−7)
Δ9
18:1 (n−9)
oleic
Δ6
18:2 (n−9)
E
20:2 (n−9)
Δ5
20:3 (n−9)
mead
E
22:3 (n−9)
diet
18:2 (n−6)
linoleic
Δ6
18:3 (n−6)
γ − linolenic
E
20:3 (n−6)
dihomo− γ −
linolenic
Δ5
20:4 (n−6)
arachidonic
E
22:4 (n−6)
diet
18:3 (n−3)
α −linolenic
Δ6
18:4 (n−3)
E
20:4 (n−3)
Δ5
20:5 (n−3)
timnodonic
E
22:5 (n−3)
Δ4
22:6 (n−3)
Δ9,6,5,4:
desaturases
E: elongases
fatty acid family:
(n−9)
(n−6)
(n−3)

The role of (n-3) fatty acids in vision has been studied by Anderson and Maude (1971) who raised rats on a fat-free diet for 10 weeks, adequate time for several membrane turnovers. All other tissues reflected an impact of diet (indicated by an increased content of 20:3 (n-9), typical of essential fatty acid deficiency), except retinas, which conserved (n-3) content, in the 2-position of rod outer segments. Experiments in second generation rats on a fat-free diet demonstrated a specific reduction in DHA, from 45 to 19 mole%, which corresponded to a functional alteration on electroretinograms (Benolken, Anderson and Wheeler, 1973). An essential functional requirement for (n-3) fatty acids in photoreceptor membranes has been demonstrated in deficient infant rhesus monkeys (Neuringer, Connor, van Petten and Barstad, 1984). The offspring of these monkeys, also deprived of (n-3) fatty acids during prenatal and postnatal development, but sufficient in (n-6), showed plasma depletion of DHA to 6% of controls fed ample 18:3 accompanied by an impaired visual acuity of 50%. Tinoco, Williams, Hincenbergs and Lyman (1971) were unable to demonstrate classical deficiency symptoms, impaired growth or reproduction, in third generation linolenate deficient rats; however, the tenacious retention of the (n-3) fatty acids in certain tissues: heart, liver and brain was observed. Evidence of an essential role of (n-3) in neurological activity was provided by Lamptey and Walker (1976). Rats fed, for two generations, either safflower (very low linolenate) or soybean oil (moderate linolenate) exhibited no differences except for performance on a learning impairment, Y-maze discrimination test. Lower scores were correlated to a brain phospholipid DHA content of 10 to 20% that of rats fed soybean oil.

Evidence of a human essential requirement for (n-3) was derived from a patient on intravenous hyperalimentation with a high content of linoleic but a minimal content of linolenic acid (ratio of 115:1); neurological abnormalities were corrected by administration of parenteral soybean oil (ratio of 7:1) (Holman, Johnson and Hatch, 1982). The tenacious retention, coupled with implicit transport and incorporation into particular phospholipids in specific membranes, supports a special role for (n-3) fatty acids.

The above studies were aimed at investigating the response of tissue fatty acyl composition to variation in dietary fat due to concern for essential fatty acid deficiency and its correction. Interest has since developed in the effects of feeding levels of polyunsaturates in excess of requirements for beneficial prophylactic effect against heart disease (Stubbs and Smith, 1984); with the realization that vascular plaque is comprised of lipid deposits from the blood and that blood lipids are altered by dietary lipids. In particular, growing interest in (n-3) fatty acids for protection against coronary heart disease (CHD) has led to investigations of the response of tissues to (n-3)-rich marine based diets, in lieu of linoleic, the predominant polyunsaturate (PUFA) in Western diets (Anonymous, 1985). Diets enriched with (n-3) fatty acids have been shown to reduce the size and sequelae of cerebral (Black, Culp, Madison, Randall and Lands, 1979) and myocardial (Culp, Lands, Lucchesi, Pitt and Romson, 1980) infarction <u>in situ</u> in experimental animals.

## ii: Physiological Functions of (n-3) Fatty Acids

### a) Structural Components of Biomembranes

The most important role of essential fatty acids is as components of glycerophosphatides of all mammalian membranes (Sinclair, 1980). The fatty acids in the 2-position of phospholipids reflect: i) the fatty acid composition of dietary fats (Goodnight, Harris, Connor and Illingworth, 1982), ii) their modification in the liver by desaturation and elongation, iii) selective fatty acid acylation into specific phospholipids, iv) individual tissue specificities for further modification and v) subsequent utilization (beta-oxidation, eicosanoid biosynthesis) (Hansen, 1983; Weiner and Sprecher, 1984). While present in bilayers, interacting with phospholipid headgroups, cholesterol and proteins, in a fluid-mosaic conformation, fatty acids are also involved in homeoviscous control (Mead, 1984). Compositional alterations, in chain length, in degree of unsaturation or in isomerization, exert control over intrinsic enzymes of the membranes and integral proteins, via membrane fluidity, permeability, receptor-binding and transport processes (Demediuk and Harrocks, 1984). Such influences have been observed with erythrocyte passive sugar transport protein (Tefft, Carruthers and Melchior, 1986) and with ($Na^+$-$K^+$)-ATPase (Ahmad and Leeds, 1985).

### b) Eicosanoid Biosynthesis

From the average intake of arachidonic acid (AA) of 200 mg per day, the production of eicosanoids accounts for 1 mg; representing $10^{-4}$% of the potential membrane precursor pool converted to eicosanoids (Kinsella,

1986). The balance of 199 mg is thus available for membrane structure, function and other processes (Davidson and Cantrill, 1985).

Eicosanoid, is a general term for the oxygenated, biologically active derivatives of the 20-carbon fatty acids of the (n-6) and (n-3) families; forming prostaglandins (PG), thromboxanes (TX), prostacyclins (PGI) and leukotrienes (LT) of the 1-, 2-, and 3- series, respectively. They are autocoids, important local chemical mediators that 'hormonally' control many physiological and biochemical functions, such as regulation of vascular tone, permeability of capillaries and venules, contraction and relaxation of muscles, stimulation or inhibition of platelet function, activation of leukocytes, regulation of renal blood flow and mineral metabolism (Marcus, 1984). By altering the type of fat consumed, the types and amounts of eicosanoids produced can be altered (Higgs, 1985; Salmon and Terano, 1985).

Phospholipase $A_2$ liberates the fatty acid substrates dihomo-gamma-linolenic (DGLA), arachidonic (AA) and eicosapentaenoic (EPA) acids from biomembranes. When acted upon by cyclooxygenase (E.C. 1.14.99.1), which has an affinity for AA over DGLA and EPA, endoperoxide intermediates are formed (Hamberg, Svensson Wakabayashi and Samuelsson, 1974). In platelets, AA is transformed to thromboxane $A_2$ ($TXA_2$) which has strong platelet aggregating and vasoconstricting effects (Hamberg, Svensson and Samuelsson, 1975). In vascular endothelium, AA is converted to prostaglandin $I_2$ ($PGI_2$), also known as prostacyclin, a vasodilator and potent inhibitor of platelet aggregation (Moncada, Gryglewski, Bunting and Vane, 1976). The balance between $TXA_2$ and $PGI_2$ regulates the initial steps of platelet aggregation and is thought to play a role in the

pathogenesis of vascular disorders. While DGLA possesses antiaggregatory properties, due to its low availability, poor suitability for thromboxane synthetase (Willis, 1981) and lack of conversion to 1-series prostacyclins (Dyerberg and Bang, 1980), it provides little competition for 2-series eicosanoid synthesis. Aspirin can inhibit the formation of 2-series endoperoxides by acetylation of the cyclooxygenase active site, which is effective for the 7 to 10 day lifespan of the platelet (Moncada and Vane, 1978) but eliminates both $PGI_2$ and $TXA_2$ production. When the diet is supplemented with fish or fish oil, EPA and DHA can also competitively inhibit the enzymes governing AA metabolism. This results in the production of $TXA_3$ by platelets and $PGI_3$ by vessel walls (Needleman, Minkes and Raz, 1976; Needleman, Raz, Minkes, Ferrendelli and Sprecher, 1979). $TXA_3$ has less potent platelet aggregating effects than $TXA_2$ and $PGI_3$, like $PGI_2$, is an effective antiaggregating substance. While $PGI_3$ has been shown to be induced by diet in man (Fischer and Weber, 1984); its ingestion does not inhibit the synthesis of $PGI_2$, contrarily, it adds to net prostacyclin production in a stoichiometric dose-dependent manner (Dyerberg, Jorgensen and Arnfred, 1981). The net result is an antithrombotic state.

In addition to their effects on platelets and vascular endothelium, (n-3) fatty acids alter the production of leukotrienes by lipoxygenases (E.C. 1.13.11.12) in leukocytes and neutrophils (Hansson, Lindgren, Dahlen, Hedqvist and Samuelsson, 1981). Formed from DGLA, AA and EPA, leukotrienes mediate and modulate the symptoms associated with inflammatory and hypersensitivity reactions. Pentaene leukotrienes are less effective leukotactic agents than their tetraene counterparts (Lee,

Hoover, Williams, Sterling, Ravalese, Spur, Robinson, Corey, Lewis and Austen, 1985).

c) Regulation of Lipoproteins

The third function of essential fatty acids is the transport and oxidation of cholesterol. In lipoproteins, 80% of the cholesterol is esterified (Sinclair, 1980); the type of substituent fatty acid has an impact on cholesterol mobilization, transport, turnover and excretion. Addition of polyunsaturated fat to the diet has a pronounced hypolipidemic effect (Simpson, Barker, Carter, Cassels and Mann, 1982; Lassere, Mendy, Spielmann and Jacotot, 1985).

## iii: Epidemiological Studies

Interest was stimulated by the observation that Greenland Eskimos, whose traditional diets are high in marine animals and fish, have a very low incidence of CHD, despite the highest recorded intakes of fats (Bang and Dyerberg, 1980). It is still unclear why fish or fish oil consumption is beneficial in preventing CHD but this may be explained by the effect of fish consumption on lipid, eicosanoid and lippprotein metabolism (Dyerberg, Bang, Stoffersen, Moncada and Vane, 1978). Compared with Western populations, Eskimos have lower plasma lipid levels (total, VLDL, LDL, and triglycerides) (Bang, Dyerberg and Nielson, 1971; Anonymous, 1983), elevated HDL (Dyerberg and Bang, 1979a) and decreased frequency of hypertension (Davidson and Liebson, 1986). Mortality from CHD in Eskimos was 3.5% and in Danes 50% (Dyerberg, 1981). Both populations had equal intakes of fat whereas the daily intake of cholesterol in Eskimos was 245 and in Danes 139 mg/1000 calories (Bang,

Dyerberg and Hjorne, 1976). A similar dietary intake of polyunsaturated versus saturated (P/S) fats could not explain the lower serum cholesterol levels in Eskimos (Dyerberg and Bang, 1979a). These characteristics were not genetic in origin but resulted from the Eskimo diet (Dyerberg and Bang, 1979b) of cold-water fish (mackerel, salmon, herring and cod), seal and whale meat (Davidson and Liebson, 1986). A shift in the source of PUFA from (n-6) to (n-3) fatty acids provided 5.8 g/day (n-3) in Eskimos[1] versus 0.8 g/day in Danes (Goodnight et al., 1982). Platelet phospholipids reflected dietary fatty acid patterns, indicating a shift in the AA:EPA ratio from 40:1 in Danes to 1:1 in Eskimos (Dyerberg et al., 1978, 1979b). Haemostasis is reflected in vivo by bleeding time, which is dependent on the balance between the proaggregatory TXs in the platelets and the antiaggregatory PGIs in the vascular tissue. Eskimos had a bleeding time of 8.05 minutes compared to 4.76 in Danes (Dyerberg and Bang, 1979a). Such observations disputed the hypothesis that a high intake of fat was the primary cause of CHD and led to the hypothesis that large quantities of (n-3) fatty acids protected Eskimos against the thromboembolic complications of cardiovascular disease. These benefits were attributed to an imbalance between the antagonistic effects of TXs and PGIs induced by triene eicosanoids on haemostasis.

Epidemiological studies in Japan have also been of interest, due to a diet rich in fish but low in fat (Sinclair, 1980). Yamori, Nara, Iritani, Workman and Inagami (1985) examined the serum fatty acids from phospholipids in inhabitants of a Japanese fishing village (fish

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[1]Sanders (1985) reported the Eskimo daily intake of EPA of 5 g and DHA of 6 g.

consumption 250 g/day) to age-matched males in a Japanese farming village (fish consumption 90 g/day) and an inland population (Tennessee) in the United States, where death rates from CHD were six times higher than in Japanese populations. The AA levels were not found to be different. EPA levels, while half the level found in Eskimos, were four times that found in Americans. DHA levels were twice as high as Eskimos and nine times that of Americans. The Japanese ratio for (n-3)/(n-6) was seven to ten times that determined in the Americans. Differences between the two Japanese groups were found in DHA and in the ratio of (n-3)/(n-6). A lower stroke morbidity was associated with the seaside diet.

Kromhaut, Bosschieter and de Lezenne Coulander (1985) calculated the relationship between fish intake and coronary deaths for a 20-year period in the Netherlands. Fish consumption was found to be an independent negative risk factor for CHD, in a sample of 852 middle-aged men. Mortality from CHD was more than 50% lower among those who consumed at least 30 g fish/day. These risk ratios revealed that even modest fish intake was beneficial, compared to relatively large intakes found in Eskimos (400 g/day) or in Japanese (100 g/day). However, this study also exemplified the need for controlled prospective studies examining the effect of fish on CHD, as well as the need for determining the smallest effective dose.

**iv: (n-3) and (n-6) Antagonism**

Since the balance between $TXA_2$ produced by the platelet and $PGI_2$ from vascular tissue plays an integral role in many pathophysiologies, any change in (n-6) metabolism is crucial. An impact of (n-3) fatty

acids on (n-6) has been demonstrated in their common physiological roles as structural components of membranes, precursors of eicosanoid biosynthesis and in regulation of lipoproteins.

Intestinal absorption and lymphatic transport of 20:5 (n-3) was found comparable to both 18:1 (n-9) and 20:4 (n-6) (Chen, Subramaniam, Cassidy, Sheppard and Vahouny, 1985). When Iritani and Fujikawa (1982) studied (n-3) incorporation into phospholipids of rat tissues, the (n-3) fatty acids were found to be comparably incorporated when provided alone or in equal portions with (n-6). Total unsaturation was strictly maintained as were the levels of fatty acids with more than three double bonds. It is believed that the specificity of phospholipid acylating enzymes for C-1 and C-2 positions of the glycerol molecule limited the PUFA content to less than 50% (Lynch, Locicero and Schneeberger, 1986). When both LA and LNA were provided, acyl CoA synthetases exhibited comparable selectivity for (n-3) and (n-6) fatty acids; desaturases favoured (n-3) (Brenner and Peluffo, 1966) but acyltransferases favoured (n-6) (Iritani, Ikeda and Kajitani, 1984).

Corroboration of these findings was provided by Weiner and Sprecher (1984). Feeding 18:2, 18:3 (n-3) or equal amounts of both to rats, there was no alteration of the saturate content (16:0, 18:0) in platelets or liver phospholipids. Linoleate alone caused no alteration in AA but partially replaced (n-3), and in particular, 22:5 (n-6) for 22:6 (n-3). Linolenate enhanced the selective acylation of EPA, which was accompanied by depressed levels of AA. When the diet contained both PUFAs, a selective conversion of LA to AA and its acylation versus the analogous metabolism of LNA occurred. In a time-course study on rats, substitution

of (n-9) at the C-2 position of liver, plasma and platelet phospholipids was accomplished by both (n-6) and (n-3) diets, in six days (Iritani and Narita, 1984).

Marshall and Johnston (1982) and de Schrijver and Privett (1982) found that with feeding various increments of LNA to LA to rats, the elongated and desaturated metabolites of the (n-6) series decreased while (n-3) metabolism increased, reciprocally. The replacement of (n-6) by (n-3) was attributed to the effective competition of LNA over LA for delta-6 desaturase. This was evidenced by a depression of PG-2 synthesis by competitive inhibition of PG synthetase subsequent to decreased levels of substrate AA, in a tissue-specific manner.

LNA has a potent impact on 2-series PG substrate availability including its elongation and desaturation. On the other hand, EPA can lower both substrate availability and competitively reduce (to 2/3) the 2-series prostaglandins. Hwang and Carroll (1980) who fed methyl linolenate[2] to EFA-deficient rats, found a diet-induced increase of (n-3) fatty acids in liver and in serum lipids and serum concentrations of PG and TX of the 2-series were attenuated. The simultaneous provision of LA demonstrated an inhibited conversion to AA and decreased PG generation with increasing levels of LNA.

Using a marmoset monkey model, McMurchie, Gibson, Charnock and McIntosh (1986) found the major (n-6) PUFA to undergo change was 18:2; while 22:5 and 22:6 were the most labile (n-3)s. Preferential desaturation of (n-3) was observed although compensatory variation in DHA in response to oscillating levels of (n-6) in plasma, red blood cell

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[2]and constant methyl linoleate

(RBC), liver, heart and kidney occurred in an 18:2-rich diet. Despite a disparity in the quantity of PUFA provided, the level of tissue unsaturation remained constant, as previously seen in the rat (Gibson, McMurchie, Charnock and Kneebone, 1984). The authors indicated that this was due to a homeostatic mechanism which maintained a constant proportion of saturation in the membrane, despite the diet, to stabilize membrane fluidity. Although monounsaturation contributes to unsaturation, by far the greatest contributors are (n-6) and (n-3) fatty acids. In the marmoset, unlike the rat, the proportion of AA actually decreased in plasma, RBC, liver, heart and kidney despite a significant incorporation of LA from the diet. This result points to a species difference; whereby an induced elevation of LA may have led to an inhibition of delta-6 and delta-5 desaturase and/or elongase activity with a subsequent decrease in AA. Brenner (1969) had however, observed mutual inhibition of desaturation in rats, indicating that DGLA desaturation to AA competes with 18:2 desaturation to 18:3 (n-6) and vice versa.

The preponderance of (n-3) as long chain homologues, 22:5 and 22:6, while (n-6) fatty acids mainly occur as 18:2 and 20:4, although both are supplied as 18-carbon precursors, demonstrates the preference of delta-4,5,6 desaturases and specificity of C18- and C20-elongation enzymes for (n-3) rather than (n-6) fatty acids (Hagve and Christophersen, 1984; Stubbs and Smith, 1984).

The antagonism of (n-3) and (n-6) fatty acids includes more than competitive incorporation into tissues. Hirai, Terano, Hamazaki, Sajiki, Kondo, Ozawa, Fujita, Miyamoto, Tamura and Kumagai (1982) proposed that (n-3) fatty acids exert their influence at the rate-limiting step of

release from membrane, by phospholipase $A_2$. Other evidence indicated the competitive inhibition of cyclooxygenase and of lipoxygenase (Needleman et al., 1976, 1979; Hornstra, Haddeman and ten Hoor, 1979; Culp et al., 1980).

Although EPA was readily incorporated into tissue lipids, it was a poor substrate for cyclooxygenation, being converted only 1/8 or 92% less efficiently than AA (Needleman et al., 1979; Marcus, 1984). However, EPA had a high binding efficiency for cyclooxygenase and thus competed with AA, inhibiting its conversion to proaggregatory eicosanoids (Whitaker, Wyche, Fitzpatrick, Sprecher and Needleman, 1979).

The capacities of EPA and DHA to antagonize metabolism of AA by the 5-lipoxygenase pathway of human leukocytes and neutrophils are distinctly different. DHA does not interfere with AA and is not converted to a leukotriene. EPA competitively inhibits the generation of tetraene leukotrienes by epoxide hydrolase, preferentially producing pentaene leukotrienes, which have attenuated chemotactic activity (Lee, Mencia-Huerta, Shih, Corey, Lewis and Austen, 1984).

The differences in the lipid-lowering activity of (n-6) and (n-3) were recognized by the study of Harris, Connor and McMurry (1983a), comparing salmon oil (20% n-3) with vegetable oil (54% n-6), in normolipidemic subjects. Within four weeks, the study demonstrated an equally significant fall in plasma and LDL cholesterol in both groups but only the salmon oil group experienced an additional reduction in triglyceride and VLDL portions. On a gram-for-gram basis, the (n-3) diet had a greater hypocholesterolemic effect than (n-6). The decrease in plasma cholesterol was proportional to the degree of unsaturation; (n-3)

diet provided 2.75 times more unsaturation than the vegetable oil diet. However, hypotriglyceridemia did not correlate with the degree of unsaturation and was attributed to either increased catabolism of VLDL or to inhibition of its synthesis/secretion. This second hypothesis was verified when EPA, not LA, was shown to reduce the availability of fatty acid substrates for the lipogenic activity of acetyl CoA carboxylase in rats (Iritani, Inoguchi, Endo, Fukuda and Morita, 1980) and when reduced triglyceride levels were ascertained in fat absorption tests (Saynor, Verel and Gillot, 1984).

## v: Human Clinical Studies

The results of human feeding trials have given disparate results due to the subject selection, the type of fish oil/fish/concentrate used, the dosage, the duration and the attention given to simultaneous intake of (n-6) antagonists and cholesterol. It is therefore difficult to extrapolate the results from one study to another.

a) Plasma Lipids

In many clinical trials, investigators supplemented with doses of 4 to 5 g EPA/day to emulate the quantities consumed by Greenland Eskimos. Manipulation of fatty acid composition was demonstrated in human plasma phospholipids although the magnitude varied, especially with dose and duration. Decreases in LA content and increases in the level of EPA, often greater than those of DHA, were noted (Bronsgeest-Shoute, van Gent, Luten and Ruiter, 1981; Brox, Killie, Gunnes and Nordoy, 1981; Fischer and Weber, 1983; Harris, Rothrock, Inkeles, Illingworth, Connor and Goodnight, 1983b; Lorenz, Spengler, Fischer, Duhm and Weber, 1983;

Illingworth, Harris and Connor, 1984; Popp-Snijders, Shouten, de Jong and van der Veen, 1984; Simons, Hickie and Balasubramaniam, 1985; von Schacky, Fischer and Weber, 1985a; Thorngren, Nilsson and Gustafson, 1986). These findings suggested that EPA substituted preferentially for LA (Skeaff and Holub, 1986). The AA content of plasma lipids appeared to be more resistent to dietary changes. Several authors noted how rapidly changes in fatty acids occurred, although more pronounced changes were found with a lengthened experimental period. Previously, Farquhar and Ahrens (1963) contested that such rapid changes in lipid profiles renders plasma a poor model for dietary study.

b) Plasma Lipoproteins and Triglycerides

Several of the studies of plasma phospholipids showed a simultaneous manipulation of plasma lipoproteins. Decreases in plasma VLDL and triglycerides (TG) were observed in most human feeding trials. Feeding of linseed (Sanders and Roshanai, 1983), sardine (Hirai et al., 1982) and herring (Singer, Jaeger, Wirth, Voigt, Naumann, Zimontkowski, Hajdu and Goedicke, 1983) oils, all which contained very little EPA, did not decrease plasma TG to the magnitude observed for cod liver oil (CLO) (Sanders, Vickers and Haines, 1981; Lorenz et al., 1983; von Schacky et al., 1985a), MaxEPA[3] (Saynor and Verel, 1980, 1982; Sanders and Hochland, 1983; Sanders and Roshanai, 1983; Saynor et al., 1984; Simons et al., 1985; Nestel, 1986), SuperEPA[3] (Popp-Snijders, Shouten, van Blitterswijk and van der veen, 1986a), salmon (Harris et al., 1983a; Illingworth et al., 1984; Phillipson, Rothrock, Connor, Harris and Illingworth, 1985), mackerel (von Lossonczy, Ruiter, Bronsgeest-Shoute, van Gent and Hermus,

[3]a proprietary fish oil concentrate

1978; Singer et al., 1983; Thorngren et al., 1986) or fish oil concentrates (van Gent, Luten, Bronsgeest-Shoute and Ruiter, 1979; Bronsgeest-Shoute et al., 1981). Low erucic rapeseed oil, containing LNA, did however, decrease TG by 22% in a relatively long-term study (Lasserre et al., 1985). In MaxEPA feeding trials, decreases ranged from 8% when 1.7 g EPA and 1.2 g DHA were consumed daily for 2 weeks (Sanders and Hochland, 1983), to 65% when 13.2 g of (n-3) were provided for 3 weeks (Nestel, 1986). Dose-response studies all demonstrated a proportional reduction of TG to the supplemental dose and were reversible upon cessation of fish oil ingestion (van Gent et al., 1979; Bronsgeest-Shoute et al., 1981; Sanders and Roshanai, 1983; Singer et al., 1983; Simons et al., 1985). Three studies involving hyperlipidemic subjects exhibited the greatest hypotriglyceridemic effects (Saynor et al., 1984; Phillipson et al., 1985; Simons et al., 1985).

The effect of dietary (n-3) on the plasma cholesterol levels also appeared to depend on both the type and the dose. Significant reductions were only incurred in studies where greater than 3.3 g EPA (Sanders and Roshanai, 1983), 3.6 g LNA (von Lossonczy et al., 1978; Lasserre et al., 1985) or 13 g (n-3) (Harris et al., 1983a; Illingworth et al., 1984; Phillipson et al., 1985; Nestel, 1986) were provided daily. The most pronounced results were observed when salmon oil and/or hypertriglyceridemic subjects were involved. Changes in HDL-cholesterol concentrations were not consistently observed in human feeding trials. Maximal increases in HDL were incurred with MaxEPA (Saynor and Verel, 1980) at 20 ml/day for 5 weeks or with 6.5 g (n-3) for 2 weeks (Sanders and Roshanai, 1983). Any decreases in total cholesterol were not likely

attributable to the HDL-portion. An increase in HDL is suggestive of an enhanced removal of cholesterol; whereby (n-3) fatty acids act by shifting body cholesterol pools from the tissues to the serum. Apparently this is not the mechanism by which (n-3) act on total cholesterol (Saynor et al., 1984).

Reduction of total cholesterol levels is often consistent with VLDL and LDL levels. Because of the precursor-product relationship of VLDL and LDL, a fall in LDL occurs with a much reduced synthesis of VLDL. Manipulations of VLDL and LDL are dose-related, where 4 to 8 g/day (n-3) were sufficient to reduce synthesis of apoprotein B (von Schacky et al., 1985a). Illingworth et al. (1984) noted a 30% decrease in synthesis of apoprotein B but no reduction of its fractional catabolic rate. However, Thorngren et al. (1986), using a more moderate (n-3) intake, did not detect a change in apo-A nor -B levels nor a decrease in total cholesterol, despite the incorporation of (n-3) in the plasma and platelets. Hypertriglyceridemic subjects showed a marked response in VLDL and LDL levels. Phillipson et al. (1985) asserted that the most likely mechanism for the hypotriglyceridemic action of (n-3) in these subjects is the depression of VLDL and LDL synthesis, improved conversion of VLDL to LDL and enhanced fecal steroid excretion.

Beitz, Hoffman, Taube, Beitz and Forster (1985) proposed a mechanism of action for the role of elevated LDL levels in the pathogenesis of atherosclerosis. Human-LDL perfused rat aortas enhanced the formation of $TXA_2$ and inhibited the formation of $PGI_2$, thereby increasing the ratio of $TXA_2/PGI_2$. HDL, on the other hand, did not alter this ratio, at higher concentrations, it has stimulated $PGI_2$ formation. LDL as peroxide

carriers may also act to inhibit $PGI_2$; while HDL, exports peroxides from the same site. Further study of lipoprotein action on $TXB_2$ formation by platelets, showed a positive correlation between LDL and $TXB_2$ formation and a negative correlation for HDL and $TXB_2$ (Beitz, Block, Beitz, Muller, Winkler, Dargel and Mest, 1986). These results suggested that the proatherosclerotic action of elevated LDL may mediate eicosanoid metabolism.

c) Platelets

Platelets, with 77% of lipid present as phospholipids, (Ikeda, Shimada and Matsumoto, 1984) are also labile to dietary manipulation. Unlike erythrocytes, platelets can synthesize fatty acids and phospholipids de novo (Ikeda et al., 1984; Valles, Aznar and Santos, 1984; Weiss, 1975). Significant increases of platelet EPA and DHA content have been incurred when CLO (Brox et al., 1981; Sanders et al., 1981; Lorenz et al., 1983; Ahmed and Holub, 1984; von Schacky et al., 1985a; von Schacky, Seiss, Fischer and Weber, 1985b), MaxEPA (Sanders and Younger, 1981; Sanders and Roshanai, 1983; Simons et al., 1985) and mackerel (Seiss, Roth, Scherer, Kurzmann, Bohlig and Weber, 1980) were fed to humans. EPA, alone, increased in response to linseed, sardine oils or its concentrate (Sanders and Younger, 1981; Hirai et al., 1982; Sanders and Roshanai, 1983; Ikeda et al., 1984; Galloway, Cartwright, Woodcock, Greaves, Graham, Russell and Preston, 1985); thereby providing evidence of the platelet's capacity to convert LNA to EPA but not DHA. A concurrent decrease was observed for LA, 22:4 (n-6) and especially AA, resulting from a competitively inhibited synthesis by (n-3) (Sanders and Roshanai, 1983). The AA content was not however, diminished with linseed

(LSO), sardine oils or an EPA concentrate. Despite the fish oil source, the platelet EPA:AA ratios unanimously reflected an impact of diet. Intakes as low as 0.8 g/day led to a significant increase in platelet EPA (Sanders and Roshanai, 1983) although maximal changes occurred with a lengthened supplementation period or with an increased dose. Changes in platelet lipid composition occurred at a slower rate than plasma (von Schacky et al., 1985a) but within one week (Seiss et al., 1980). Plasma fatty acid profiles determine those of platelets by providing fatty acids directly or entire phospholipid molecules (Valles et al., 1984). Hence plasma fatty acid profiles correlate with platelet's (r=0.70, Simons et al., 1985) and to changes in plasma induced by diet (r=0.83, Thorngren et al., 1986). It is uncertain to what extent platelet fatty acid composition must be altered to affect function but it has been suggested that PI (phosphatidyl inositol), in particular, must be changed in order to alter platelet eicosanoid production (Weiner and Sprecher, 1984). Results indicated that the incorporation of (n-3) into platelet membrane subclasses was regulated with a high degree of specificity. Provision of (n-3) did not markedly alter PI; rather, alterations were attributed to the PE (phosphatidyl ethanolamine) and PC (phosphatidyl choline) fractions (Brox et al., 1981; Fischer and Weber, 1983; Ahmed and Holub, 1984; Galloway et al., 1985; von Schacky et al., 1985b). Since PI is a major source of fatty acids for eicosanoid synthesis, the beneficial effects of EPA cannot be explained on the basis of its incorporation and mobilization in the platelet membrane (Galloway et al., 1985).

Decreased platelet counts have also been observed in subjects ingesting (n-3) fatty acids. This thrombocytopenia appeared to be

transient (Saynor et al., 1984) and was associated with larger doses (Hay, Durber and Saynor, 1982). Longer term studies indicated that it was a temporary phenomenon (Saynor et al., 1984). Hay et al. (1982) ascribed the decrease in platelet counts to reduced thrombopoiesis rather than enhanced peripheral destruction since platelet survival time has been shown to increase.

The prolongation of bleeding time (BT) represents an attenuated response to vascular injury by a shift in the platelet component of haemostatic balance. The hypothesis was that enrichment with EPA should prolong the BT by reducing platelet aggregation; which, in turn, should be related to changes in platelet and plasma fatty acid composition (Thorngren and Gustafson, 1981). In human feeding trials, increased BT has also been observed. Diets providing 1.8 to 10 g EPA/day in fish, CLO and MaxEPA, have increased mean BT by 33 to 41% (Sanders et al., 1981; Thorngren and Gustafson, 1981; Lorenz et al., 1983; Fischer and Weber, 1984; Knapp, Reilly, Alessandrini and Fitzgerald, 1986). Other investigators have obtained larger increases: 51% when 13.3 g EPA/day (Harris et al., 1983b), 81% when 30 ml CLO/day (Ahmed and Hclub, 1984) or 100% when 3.6 g EPA/day (Saynor et al., 1984) were provided. Others, however, have failed to observe any change with 1.0 to 2.9 g EPA/day (Saynor et al., 1984; Simons et al., 1985) or 25 ml CLO/day (Brox et al., 1981). Although Sanders and Roshanai (1983) did measure an increase in BT, the results did not follow a dose-response trend nor did they parallel platelet EPA incorporation. Simons et al. (1985) calculated that the correlation between BT and platelet EPA incorporation was 0.41. Since platelet aggregation was not affected by platelet fatty acid

composition nor $TXA_2$ production nor BT, it was concluded that (n-3) do not delay haemostasis (BT) as hypothesized (Sanders et al., 1981; Sanders and Roshanai, 1983; Thorngren, Gustafson and Wohlfart, 1983a; Thorngren, Shafi and Born, 1983b,d). Some other mechanism besides impaired platelet aggregation is required to explain the increase in BT since the impact on platelet function and BT assays, while similar to that of Eskimos, could not explain the findings.

Determination of BT by the Simplate technique is one of the most reproducible parameters of haemostatic effect in use. It does not however, provide information about the rate or volume of the blood loss. Thorngren, Shafi and Born (1983c) adapted the BT assay to allow for the quantification of the volume of blood, as the 'bleeding time blood volume, BTBV'. Their assays demonstrated that the blood loss in the first two minutes invariably increased and was not affected by a fish diet. After three weeks on a fish diet, when BT was not yet affected, BTBV rates were decreased; however, by the sixth week, BTBV were not significantly different from controls. BTBV were found to correlate with BT ($r=0.77$). Aspirin was found to increase both BT and BTBV in a manner distinct from a fish diet despite the similarities in their effects on platelet aggregation. Since the combination produced additive effects, it is possible that BT and BTBV cannot be solely explained by altered balance of eicosanoids (Thorngren et al., 1983a,c).

Much of the evidence of the beneficial effects of (n-3)s is inconsistent with respect to platelets and the generation of eicosanoids. A common opinion is that the most beneficial effects are derived from the displacement of endogenous (n-6) fatty acids in membranes rather than

alteration of eicosanoid production (Bronsgeest-Shoute et al., 1981; Needleman, Wyche, le Duc, Sankarappe, Jakschik and Sprecher, 1982; Morita, Saito, Chang and Murota, 1983).

d) Erythrocytes

It has been well established that the fatty acid composition of the human erythrocyte is influenced by the diet and that the lipid is confined to the membrane structure (Horwitt, Harvey, Century and Witting, 1961). Plasma contains approximately 40% of its fatty acids as triglycerides (Valles et al., 1984); but in the RBC, less than 3% of the fatty acids are not in phospholipids (Farquhar and Ahrens, 1963). These fatty acids, unlike those of plasma and platelets, respond slowly to dietary changes, with half-times in orders of weeks (Lea, Jones and Hamilton, 1982).

Other aspects also make red cells attractive for study: i) red cells, along with plasma, supply other tissues with fatty acids for local eicosanoid and membrane synthesis (Rao, Siler and Larkin, 1978), ii) a deficiency of acetyl-CoA carboxylase renders the mature red cell incapable of de novo fatty acid biosynthesis from acetate (Pittman and Martin, 1966), iii) no fatty acyl chain elongation nor desaturation is performed by the red cell (Lea et al., 1982), iv) the cell has no capacity to synthesize sterols, phospholipids (Farquhar and Ahrens, 1963) nor protein (Winterbourn and Batt, 1970). Because the red cell lacks the ability for de novo fatty acid biosynthesis, its 120-day lifespan (Wagner, Chiu, Schwartz and Lubin, 1985) corresponds to less than 1% of its total cell population undergoing removal and replacement each day (Wagner et al., 1985). The incorporation of fatty acids occurs when new

cells are formed in the long bone marrow. Farquhar and Ahrens (1963), postulated however, that with the fatty acids of the membrane reaching equilibrium with those of the diet in 4 to 6 weeks, exchange must occur between the mature cell and precursor pools, allowing incorporation of new fatty acids to occur other than at erythropoiesis. In a study comparing various rat tissues as indices of prolonged (n-3) intake, Roshanai and Sanders (1985) concluded that the RBC was the best index. Only the platelet and the RBC fractions were found to accumulate EPA. EPA only became a major component of platelet lipids when AA was insufficient. These tissue lipids did however accumulate DHA but significant incorporation may require months (Horwitt et al., 1961). RBC fatty acid composition has been demonstrated to reflect those of cardiac (Holman, 1960) and neural (Carlson, Carver and House, 1986) tissues.

Additional advantages of red cells as a research model are their relative abundance, accessibility and ready isolation, uncontaminated by other cells (Owen, McIntyre and Gillet, 1984). The lipid of the cell resides in its plasma membrane. Therefore the analysis of intact cells pertains to the membrane lipid and is preferable since lipid loss can be incurred in the preparation of red cell ghosts (Turner and Rouser, 1974).

It is for the above reasons that the fatty acid composition of RBC membranes has been studied even though haemostasis is considered a function of platelets. RBC fatty acid patterns have been studied for indications of predisposition to coronary thrombosis in humans; erythrocyte EPA content was inversely related to thromboses (Lea et al., 1982; Simpson et al., 1982). It was concluded that the RBC provides the

best available retrospective index of the types of fats ingested over preceding weeks (Simpson et al., 1982).

The four major lipid species in erythrocytes are PC, PE, SM (sphingomyelin) and PS (phosphatidyl serine), with smaller contents of glycolipids, free fatty acids, phosphatidic acid and PI. However, these cells are devoid of triglycerides and cholesterol esters. Of the phospholipids species, PC, PE and SM each comprise 25 to 30% of the total, PS, represents 10 to 15% (Schwartz, Chiu and Lubin, 1985). Lipids in the RBC plasma membrane are distributed asymmetrically between the bilayer leaflets which must serve some vital function in the maintenance of haemostasis since it is carefully maintained throughout the circulating lifespan of the cell (Schwartz et al., 1985). Each phospholipid represents a heterogeneous population of molecules differing in composition in their respective acyl moieties. The outer monolayer, enriched in choline-phospholipids, tends to contain relatively saturated fatty acids; the ethanolamine-enriched inner leaflet is relatively unsaturated. Ethanolamine phospholipids contain an elevated content of AA, 22:5 (n-6) and DHA; PS is elevated with respect to 18:0, 18:1 and AA; PC contains elevated 16:0, 18:0, 18:1 and LA; while SM is distinguished by elevated 22:0, 24:0 and 24:1 (Ways and Hanahan, 1964). PE and PS together contain an elevated amount of 22:4 (n-6), 22:5 (n-6), 22:5 (n-3) and 22:6 (n-3) (Dodge and Phillips, 1967). The ethanolamine plasmalogen component, representing 12 to 13% of the total phospholipid (Ways and Hananan, 1964), contains 93% unsaturated fatty acid, including AA, 22:4 (n-6) and DHA (Williams, Kuchmak and Witter, 1966). In total, the four

C22 fatty acids from (n-6) and (n-3) series comprise 17% of the red cell fatty acids (Horrobin, 1983).

Fatty acid composition influences the cell fluidity, which determines cell functions: flexibility, permeability, oxygen transport and enzyme and hormone receptor activities. Changes in fatty acid composition are seen as alterations in cell rheological properties. Cell deformability is an important microcirculatory parameter since the diameter of the terminal capillaries is smaller that of RBC (Hanss and Koutsouris, 1985). It is believed that such alterations of rheological properties by incorporation of (n-3) might facilitate microcirculation (Dyerberg, 1986).

Early work on the effect of diet on red cell composition was determined in rats (Walker and Kummerow, 1963, 1964). When diets that differed in unsaturated fatty acids were provided, the primary effect involved only the fatty acyl moieties of the lipids rather than the proportions of total extractable lipid, cholesterol or distribution of lipid phosphorus. This lack of effect on other than the fatty acids was previously noted by Monsen, Okey and Lyman (1962). Both groups reported that the AA content was independent of dietary fat, which may imply a more important role of this fatty acid in maintaining the physical characteristics of RBC. Its precursor, LA can vary by twofold or more; whereas the synthesis of AA occurred at the expense of LA, when dietary restrictions were imposed. In subsequent experiments, they concluded that the RBC responds to dietary fatty acid variation in a very specific manner and that alterations are subject to limitations. Fatty aldehydes,

derived from plasmalogens, were found to participate also in the diet-induced alterations (Walker and Kummerow, 1964b).

When various oils, including fish and LSO, rich in (n-3) fatty acids were fed to rabbits, the erythrocyte fatty acyl composition reflected the pattern of dietary enrichment (Housley, Born, Conroy, Belin and Smith, 1986). Once again, any effects of the oils were attributed to changes in fatty acids alone and the AA content remained constant when either (n-6) or (n-3) fatty acids were fed. In rabbits, long chain (n-3)s, EPA, 22:5 and DHA, increased only in the case of fish oil feeding; feeding LSO had no effect. Fish oil also had an impact on RBC physical properties: deformability was decreased while osmotic haemolysis was increased. LSO, however, significantly decreased osmotic haemolysis, compared to the fish oil diet. No correlation was found between the unsaturation index and RBC osmotic fragility but a highly significant relationship was found between the LA content and osmotic haemolysis. Since safflower oil gave similar results to LSO oil, the researchers postulated that alpha-linolenyl acyl groups may exert a similar influence to LA on haemolysis (Housley et al., 1986). One might explain the opposing effects of fish oil and LSO on osmotic haemolysis as dependent on the differing abilities of longer versus shorter chain (n-3)s to displace LA.

Comparison of fatty acid profiles of erythrocytes for rats, mice, hamsters, guinea pigs, rabbits and humans revealed large species differences in (n-6), (n-3) and their ratio (Horrobin, Huang, Cunnane and Manku, 1984). The 22-carbon (n-6) were much more evident in humans than other species. The exceptionally high AA content in rat RBC or low level

of LNA metabolites in rabbits and in rats infer that certain species have limited application to humans (Horrobin et al., 1984).

The emphasis in human studies involving (n-3) fatty acids has been on dietary supplements and platelet function. The erythrocytes which are indicative of dietary history need further experimentation.

Sanders et al. (1981) supplemented the diet of male volunteers with CLO for 6 weeks. The proportion of EPA, 22:5(n-3) and DHA increased remarkedly (refer to Table A) at the expense of (n-6) fatty acids, LA, AA and adrenic acid. The erythrocyte content of EPA was appreciable after one week.

Approximately double the dose of CLO was employed by Lorenz et al. (1983), in a 25-day study. The (n-3) fatty acids were rapidly incorporated into plasma, platelets and RBC fractions, once again, at the expense of (n-6), despite their continued dietary supply. In the red cell, EPA and DHA increased significantly and only LA decreased; little change was observed in the saturated and monounsaturated fatty acids. These researchers explained their findings as competition of the polyunsaturates for the 2-position of phospholipids (PL); whereby, saturates and monounsaturates, which predominantly esterify at the 1-position, would thus remain unchanged. Although the 120-day lifespan of the red cell exceeded the length of this study, the authors concluded that the fatty acid profile must equilibrate continuously with the lipid environment. Concomitantly, platelet fatty acid composition, BT, $TXB_2$ and aggregation reflected the (n-3) content of the diet. Since incorporation of (n-3) fatty acids into the red cell can influence transmembrane ion transport systems, sodium-potassium cotransport and

Table A: Human (n-3) Studies of Erythrocyte Fatty Acid Composition

| Author | Source of (n-3) | Effect on Erythrocyte |
|---|---|---|
| Sanders et al. (1981) | 20 mL/day CLO (1.8 g EPA + 2.2 g DHA)<br>6 weeks | + 236 % EPA<br>+ 24 % 22:5 (n-3)<br>+ 43 % DHA<br>- 13 % LA<br>- 12 % AA<br>- 13 % 22:4 (n-6)<br>NS 22:5 (n-6) |
| Lorenz et al. (1983) | 40 mL/day CLO (10 g n-3: 4 g EPA + 6 g DHA)<br>25 days | + 500 % EPA<br>+ 43 % DHA<br>- 20 % LA |
| von Schacky et al. (1985a) | 10-40 mL/day CLO ( 9.4 % EPA + 13.8 % DHA)<br>4 weeks 10 mL<br>4 weeks 20 mL<br>4 weeks 40 mL<br>8 weeks 20 mL | + EPA<br>+ DHA<br>- LA<br>- AA |
| Terano et al. (1983) | EPA ethyl ester (3.6 g/day EPA + 0.2 g/day DHA)<br>4 weeks | + 107 % EPA<br>- 21 % 18:0<br>NS % DHA<br>+ 30 % deformability<br>- viscosity |
| Kobayashi et al. (1981) | EPA concentrate derived from sardine oil (1.4 g/day EPA)<br>4 weeks | - 16 % viscosity |
| Cartwright et al. (1985) | MaxEPA (3.4 g/day n-3)<br>3 weeks | total/ PC/ PE/ PS[3]<br>+163/933/ 88/150 % EPA<br>+ 56/ 77/ 10/ NS % DHA<br>- 13/ 17/ 19/ NS % LA<br>NS/ NS/ NS/ NS % 22:4 |

[3]versus zero-time

| | | |
|---|---|---|
| Cartwright continued | 6 weeks | total/ PC/ PE/ PS[3] |
| | | +163/700/156/500 % EPA |
| | | + 54/ 85/ 35/ 28 % DHA |
| | | - 21/ 13/ 19/ NS % LA |
| | | - 21/ NS/ 25/ 28 % 22:4 |
| | | + deformability |
| | | - viscosity |
| Popp-Snijders et al. (1984) | 15 mL/day CLO (3 g n-3) | PC/PE |
| | 1 week | +275/ NS % EPA |
| | | + 33/ NS % DHA |
| | | - 7/ NS % LA |
| | | NS/ NS % 20:3 |
| | | NS/ NS % AA |
| | | NS/ NS % 22:4 |
| | | + 7/ 2 % DBI |
| | 2 weeks | +450/ 55 % EPA |
| | | +100/ 12 % DHA |
| | | - 13/ 5 % LA |
| | | - 19/ NS % 20:3 |
| | | NS/ NS % AA |
| | | NS/ NS % 22:4 |
| | | + 9/ 2 % DBI |
| Popp-Snijders et al. (1986a) | SuperEPA (3 g/day EPA + DHA) | + 5 % SM |
| | | - 2 % PC |
| | | - 2 % PE |
| | | PC/PE |
| | 8 weeks | +475/256 % EPA |
| | | + 77/ 34 % DHA |
| | | - 12/ 18 % LA |
| | | - NS/ 17 % 22:4 |
| | | + NS/ 26 % 22:5 |
| | | - NS/ 8 % AA |
| | | + 9/ 8 % DBI |
| Popp-Snijders et al. (1986b) | fish: herring mackerel, salmon (3 g/day n-3) | + 28 % C:P |
| | | PC/PE |
| | 4 weeks | +/+ EPA |
| | | NS/+ 22:5 |
| | | +/+ DHA |
| | | -/- LA |
| | | NS/- AA |
| | | NS/- 22:4 |
| | | + 9/4 DBI |

sodium-lithium countertransport were studied. The supplement did not influence these red cell ion fluxes.

Von Schacky et al. (1985a) supplemented CLO, in graduated doses, over an extended period of time. The incorporation of EPA and DHA in plasma, platelets and RBC followed a dose- and time-dependent pattern and increased until the termination of the study. LA and AA decreased. The sum of 16:0, 18:0 and 18:1 remained constant at 58-68%. A simultaneous shift occurred in thromboxane and prostacyclin production to a less aggregatory state. In contrast to in vitro studies, where labelled AA and EPA and some DHA are incorporated readily (Stubbs and Smith, 1984), in vivo results from this study indicated that fatty acid profiles changed with a delay, despite adequate dietary and plasma contents. Upon reduction of dose, a response of EPA and DHA was likewise delayed. The researchers concluded that these responses reflect only a limited exchange of fatty acids with plasma due to confines established at the time of cell formation. During this study, EPA and DHA increased in all phospholipid fractions; while the (n-6) exchanged disproportionately, in only the plasma and red cells. Because of this anomaly, the authors repudiated simple competition between (n-6) and (n-3) for the 2-position, as the only regulatory mechanism. Discrepancies between this and previous animal studies were attributed to greater specificity in humans.

Impaired erythrocyte deformability and increased whole blood viscosity have been noted in patients with peripheral vascular occlusive diseases; hence Terano, Hirai, Hamazaki, Kobayashi, Fujita, Tamura and Kumagai (1983), studied the effects of purified EPA on rheological properties of blood. After 4 weeks, there was a significant rise in the

proportion of EPA in plasma, platelets and erythrocytes. Interestingly, DHA did not increase in the red cell. This incorporation was associated with reduced aggregation and retention of platelets and a reduction of whole blood viscosity and an increased deformability of the red cell. The 30% increase in deformability correlated with RBC EPA incorporation ($r=0.65$), supporting the theory that the viscoelastic properties of the RBC membrane may depend on the proportion of EPA. The more EPA contained in the red cell, the more elastic was the membrane. Disordered blood rheological properties contribute to the microcirculatory disturbances in vascular occlusive diseases as does platelet hypercoagulability. It is thus possible that the co-existence of disturbed platelet and red cell functions both participate in thrombotic disorders.

The role of blood viscosity in the etiology of thrombotic disorders was studied in volunteers provided with an EPA concentrate to emulate differences previously observed between a fishing village and a farming village (Kobayashi, Hirai, Terano, Hamazaki, Tamura and Kumagai, 1981). Blood viscosity fell significantly after 4 weeks of supplementation.

Structural alterations induced in the red cell could precipitate compensatory changes to control homeostasis (Beynen, Shouten and Popp-Snijders, 1984). These authors observed increased EPA accompanied by decreased LA in red cell PC upon administration of CLO. The alteration in fatty acyl moieties was regarded as an attempt to maintain normal fluidity.

Lipid fluidity is an important factor in regulation of erythrocyte deformability. A decreased viscosity and an increased deformability was observed with a MaxEPA supplement in humans (Cartwright, Pockley,

Galloway, Greaves and Preston, 1985). Dietary MaxEPA increased the relative contents of EPA and DHA in total phospholipids after 3 and 6 weeks, almost entirely at the expense of LA. The unsaturation of total phospholipids was increased significantly in 3 weeks, seen as a 6.7% increase in the mean number of double bonds per fatty acid molecule. Mean whole blood viscosity and deformability were significantly altered in 3 weeks. Since neither plasma viscosity nor haematocrit were changed, it was likely that the effects on blood rheology were mediated by alterations in the red cell membrane fluidity.

The incorporation of EPA was not uniform in all phospholipid subclasses. After 3 weeks, incorporation of EPA into PC was greater than into PE or PS. After 6 weeks, there was no further increase in total EPA but its distribution amongst the phospholipids had changed. EPA had increased further in PE and PS but decreased in PC. Some of the differences in incorporation among the phospholipid subclasses can be explained by their respective location in the membrane bilayer. Rapid incorporation into PC pertains to its location on the outer monolayer and its renewal by direct exchange with its counterpart in plasma lipoproteins. Its rate of exchange is dependent on changes occurring in plasma, which are relatively fast. PE and PS, mostly located on the inner monolayer, rely on renewal via acylation of plasma free fatty acids into lysophospholipids. The source of these free fatty acids may well be PC, since the total EPA did not increase from weeks 3 to 6 but the EPA portion of PE and PS did. Therefore, the delayed incorporation of EPA into these fractions may have involved transacylation with a PC donor. DHA did not follow the same incorporation patterns. No significant

changes were observed for the SM fraction. SM, located on the outer monolayer and rich in saturates, seems to be resistent to this type of fatty acid supplementation, at least over the six weeks of the study. PI, as seen previously in platelets, was unchanged.

The incorporation of EPA and DHA was generally at the expense of LA in the first three weeks and LA with adrenic acid by the sixth week. Decreased LA metabolites were also noted by Sanders et al. (1981) and von Schacky et al. (1985a) but not Lorenz et al. (1983). Dietary supplementation of LA has been shown to inhibit the activity of its elongating and desaturating enzymes (Brenner, 1969). Supplementation of menhaden oil, rich in (n-3) fatty acids, depressed delta-6 desaturase activity in rats, resulting in decreased AA, 22:4 (n-6) and 22:5 (n-6) (de Schrijver and Privett, 1982). Thus, conversion and/or incorporation of (n-6) fatty acids was inhibited in this study.

Similar results were encountered by Popp-Snijders et al. (1984) using CLO in a two week study. Their purpose was to study the effect of CLO on factors characterizing fluidity in the red cell membrane using a similar dose to Cartwright et al. (1985). No changes were induced in the PL subclass distribution nor in the cholesterol:phospholipid (C:P) ratio; while changes were incurred in the fatty acid distribution and unsaturation. Incorporation of (n-3) did not occur at the same rate nor to the same degree. Again, (n-3) fatty acids rapidly incorporated into PC at the expense of LA but these alterations occurred as soon as one week. Incorporation into PE and PS occurred at a slower rate and to a lesser magnitude than PC. Both EPA and DHA increased in PE, while EPA alone increased in PS. These authors also attributed the differences in

rates of incorporation into PL to location in the bilayer. The incorporation of (n-3) into PE caused significant decline in AA and 22:4 (n-6) content subsequent to the study, indicative of inhibited conversion or incorporation of longer chain (n-6). Similar changes were not seen in the PC component since its content of these fatty acids is low. Generally, the results agreed with those of Cartwright et al. (1985), who showed slower rate of but a greater magnitude of incorporation.

The study of Popp-Snijders et al. (1986a) utilized SuperEPA, providing the same dosage of (n-3) as Popp-Snijders et al. (1984) and Cartwright et al. (1985), for an eight week period. The red cell PC fraction incorporated EPA and DHA, at the expense of LA; PE incorporated EPA, 22:5 (n-3) and DHA, at the expense of LA, AA and 22:4 (n-6). Popp-Snijders et al. (1986a) suggested that the induced increase in fatty acid composition might have been counteracted by a change in PL class distribution. Changes in either the PL class distribution or the C:P ratio have been disputed by Witting, Harvey, Century and Horwitt (1961); Farquhar and Ahrens (1963); Walker and Kummerow (1963); Iritani and Fujikawa, 1982; Popp-Snijders et al. (1984); Cartwright et al. (1985) and Housley et al. (1986). It was anticipated that (n-3) would affect the fluidity of the core more than the surface yet no effect was found by fluorescence polarization or electron-spin resonance. Possibly the increase in SM, which contains very long saturated acyl chains extending into the core of the bilayer, counteracted any manifestations of increased fluidity. Changes in fatty acid composition and unsaturation may not lead to measurable changes in fluidity, when a PUFA is substituted for another rather than a saturate. Cell flexibility,

measured as cell viscosity at low shear rates, was also unaffected and taken as further evidence of an overall negligible impact on fluidity.

When a daily supplement of fatty fish, providing an equivalent dose to previous (n-3) studies in humans, similar effects on fatty acids and deformability were ascertained in 4 weeks (Popp-Snijders, Shouten, van der Meer and van der Veen, 1986b). In contrast to previous experiments, the C:P ratio was also altered, by 28%. However, no alteration occurred in PL class distribution. Differences in the C:P ratio could not be ascribed to differences in the level of cholesterol intake between the fish and control diet; both provided approximately equal amounts of cholesterol. Based on the decrease in plasma total cholesterol and on the increase in the C:P ratio, upon removal of the fish diet, it was proposed that a diet rich in PUFA induces a redistribution of cholesterol from the plasma to the liver. In this process of redistribution, red blood cells may play a carrier role.

The increase in the C:P ratio was viewed as a mechanism for counteracting increased unsaturation. Popp-Snijders et al. (1986b) also studied viscosity as an index of cell deformability, at graduated shear rates. Measurements at elevated shear rates were believed to reflect internal fluid viscosity; those at medium to low shear rates indicated cell geometry and membrane properties. Significant findings at low shear rates were taken as an increased membrane flexibility or increased surface area to volume ratio. Upon withdrawal of the diet, PC and morphology returned to original status; while those in PE and in C:P remained significantly elevated. It was for these reasons changes in cell viscosity were ascribed to be dependent on the fatty acid

composition of the PC fraction. PC was earlier seen to be the most readily manipulated phospholipid.

## vi: Comparative (n-3) Fatty Acid Studies

Radiotracer studies *in vitro* suggest man can convert LNA to EPA and to DHA (Sanders and Younger, 1981) but this capacity has been questioned (Dyerberg, Bang and Aagaard, 1980). Consequently, the provision of EPA and DHA rather than a precursor has been more effective in changing cellular fatty acids. In a comparison of vegans and omnivores, Sanders and Younger (1981) found that vegans ingested four times more LA, but had similar intakes of total fat and LNA. The elevated dietary ratio of LA:LNA in vegans was expected to competitively suppress the extent to which LNA was converted to its metabolites. The provision of LSO at 6.5 g LNA/day for 2 weeks reduced the dietary ratio of LA:LNA from six to one in omnivores and led to a doubling of EPA in the platelet and plasma fractions. In vegans, the ratio was reduced from 16 to 3, which led to a tripling of EPA in plasma but had no effect in platelets. MaxEPA supplementation, given to omnivores, led to a four-fold increase in EPA and a lesser impact on DHA, at the expense of LA, in plasma and 22:4 (n-6) and AA in platelets. These results provided evidence that man can convert LNA to EPA, but the failure to increase DHA is consistent with the suggestion that the rate of delta-4 desaturation is low in man (Sanders and Naismith, 1979). Sanders and Younger (1981) proposed that large quantities of LA in adipose of vegans buffered short-term attempts to change the LA:LNA ratio and that more pronounced results could ensue with time. Although LSO was capable of increasing EPA levels, the

relatively smaller dose provided by MaxEPA (2.5 g/day each EPA and DHA for 2 weeks) proved more effective. These results thus illustrated that the administration of different (n-3)s was not equipotent in raising tissue levels of EPA or DHA, in short-term studies.

This was also exemplified by the study of Sanders and Roshanai (1983). In a dose-response study, even as little as 0.8 g/day of each EPA and DHA, from MaxEPA, increased the erythrocyte content of EPA, 22:5 (n-3) and DHA. The increase in 22:5 (n-3), since it was not provided extensively by the diet, was taken as evidence of chain elongation of EPA occurring in man; while the decrease in LA metabolites was interpreted as inhibited conversion. Not all LNA is destined to be converted to EPA; a large part is transferred to plasma triglycerides and is carried to utilizing tissues and fat depots. The low LNA content in all tissues, regardless of diet, might suggest it is a preferred substrate for beta-oxidation (Sanders and Naismith, 1980). A dose of 20 g/day MaxEPA was required to decrease plasma total cholesterol and to raise HDL-cholesterol levels. To lower plasma triglyceride concentration, 10 g/day MaxEPA (3.5 g C20-22 n-3) was required; the failure of LSO to change plasma triglycerides implied only long chain (n-3) fatty acids have hypotriglyceridemic properties.

**vii: Nonhuman Primate Models**

Symptoms of essential fatty acid deficiency have been induced by the absence of (n-3) fatty acids in primate diets (Fitch et al., 1961; Greenberg and Moon, 1961; Fiennes, Sinclair and Crawford, 1973; Neuringer et al., 1984). The lipid composition of monkey erythrocytes has also

been shown to be dependent on dietary fat (Fitch, Dinning, Witting and Horwitt, 1961). Compared to humans, the lifespan of the monkey red cell is shorter, 58 days (Greenberg and Moon, 1961). The C:P ratio and the distribution of phospholipid classes (Alam, 1985) is similar. Monkeys have also demonstrated a low capacity for converting LNA to DHA (Fiennes et al., 1973; Neuringer et al., 1984).

Clarkson, Adams, Kaplan and Koritnik (1984) studied non-nutritive factors influencing atherosclerosis in Cynomolgus monkeys. Their findings indicate that female Cynos, due to ovarian endocrine functioning, share with premenopausal white women, a relative protection against atherosclerosis compared to males. Gender was found to be an important factor in plasma lipid and lipoprotein profiles; the LDL fraction showed the greatest increases, while HDL was elevated in females. Von Lossonczy et al. (1978) also noted sex-related differences in human cholesterol fractions. Females exhibited a greater decrease in serum cholesterol, while males originally lower in HDL demonstrated a diet-reduced increase in HDL. Both sexes showed decreases in VLDL fractions. McMurchie et al. (1986) acknowledged the potential sex-related differences in their study, but were unable to treat their data separately according to sex. The present study was therefore designed so that males and females could be studied separately.

### viii: Peroxidative Instability of (n-3) Fatty Acids

The red cell contains elevated concentrations of PUFA, molecular oxygen and ferrous ions in the ligand state. These components might be

expected to make it susceptible to oxidative damage (Stocks and Dormandy, 1971).

Molecular oxygen can become activated by enzymatic or nonenzymatic means to form superoxide radicals, which can react with other free radicals to give rise to hydrogen peroxide ($H_2O_2$). $H_2O_2$ generated by non/enzymatic means is unstable and can be further oxidized to form hydroxy radicals, greatly facilitated by $Fe^{+2}$ (Clemens, Einsele, Remmer and Waller, 1985; Szebeni and Toth, 1986). In the absence of proper control mechanisms, free radicals, especially hydroxy, are labile to further propagation and to initiate lipid oxidation.

Among the primary targets for free radical attack are the double bonds of PUFAs; allylic hydrogens are labile to abstraction, forming lipid peroxy radicals. Lipid peroxy radicals can further react with other double bonds on the acyl chain to form a hydroperoxy ring structure. Rearrangement of the acyl structure with bond splitting results in malondialdehyde (MDA) formation.

Despite the inherent propensity for oxidation, RBCs are less prone to oxidation than other cells, due to intrinsic and extrinsic protective mechanisms (Ostrea, Cepeda, Fleury and Balun, 1985). Protection can be partly ascribed to cellular antioxidants and radical scavengers including: catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), reserves of vitamins C and E, structural compartmentalization of aminophospholipids rich in PUFA (Lubin and Chiu, 1980) and the presence of cholesterol (Szebeni and Toth, 1986). Inhibition of MDA formation was found to be proportional to the logarithm of the antioxidant concentration (Stocks and Dormandy, 1971).

The insertion of peroxide molecules at the position of fatty acid double bonds could alter acyl chain structure and impose restrictions on motion of the chains (Kobayashi, Itabe, Inoue-and Nojima, 1985). Such structural changes would thus have an impact on membrane function: fluidity, flexibility, charge, proton conductivity, permeability, haemoglobin binding, cell deformability, cell survival, transport enzymes and hypercoagulability (Jain, 1985). Large increases in the dietary polyunsaturate to saturate (P/S) ratio have been shown to increase tissue alpha-tocopherol requirements and influence their susceptibility to peroxidation as a consequence of incorporation of elevated amounts of oxidatively unstable unsaturates.

The study of Buckingham (1985) addressed the effects of various dietary P/S ratios and various doses of Vitamin E. The P/S ratios studied had no significant impact on Vitamin E requirements in a 16-week rat study. A dose of 40 I.U./kg diet Vitamin E (as alpha-tocopherol) was found adequate for maximal inhibition of lipid peroxidation at the P/S levels tested.

Because of the highly unsaturated nature of (n-3) fatty acids, the vitamin E requirements of the monkeys in this study may indeed have been increased. Although tocopherol is localized in the erythrocyte membrane (Kayden and Bjornson, 1972), deficiency of this vitamin is not expected to exert an influence on the constituent fatty acids of the RBC phospholipids (Walker and Kummerow, 1964a). Rather, depletion of vitamin E has been found to alter the red cell mass, size, sensitivity to peroxidation (Kayden and Bjornson, 1972) and survival time due to peroxide haemolysis (Horwitt et al., 1961; Monsen et al., 1962; Walker

and Kummerow, 1964a). Previous attempts to demonstrate an increased susceptibility to peroxidation with red cell counts and thiobarbituric acid have been unsuccessful (Walker and Kummerow, 1964a).

## ix: Rationale and Aims for the Present Study

The comparison of sources of (n-3) and their dietary role in humans, particularly in erythrocytes, is poorly documented. Results of many clinical studies conducted to date are considered to be of limited value due to poor experimental design. Some investigators used relatively small groups of subjects treated for short periods of time and did not conduct the trials under double-blind or placebo-controlled conditions. When humans were studied, (n-3) fatty acids were generally added as supplements to rather than incorporated into regular diets. Little attention was given to the intake of (n-6) fatty acids or cholesterol. Depending on the supplement used, some subjects were required to swallow several capsules daily; the practicality and safety of consuming such large doses is questionable. Cod liver oil or other fish liver oils should not be recommended as (n-3) supplements; these products contain significant quantities of vitamins A and D as well as cholesterol. A smaller effective dose of (n-3) remains to be determined. Long-term controlled studies should be able to establish the utility of fish oil in treating or preventing CHD and possible adverse effects.

The present study was based on the use of monkeys, rather than rodents, because of their biological relevance to humans. Also, their diets were relatively easier to control, over a prolonged period, than would be possible in a human study. Few studies have been done comparing

(n-3) fatty acids of differing chain lengths. Therefore, the present study examined the effects of short versus long chain (n-3) fatty acids in the presence of their (n-6) analogues. The erythrocyte was chosen an index because it best reflects the long-term dietary intake of (n-3) fatty acids. The feeding experiments were designed so that there was an equal provision of polyunsaturates and of (n-6) to (n-3). The fatty acid composition of the erythrocyte was examined as a function of dietary group, sex and time. In addition, peroxidative studies were conducted because of the potential for an increased susceptibility to peroxidation resulting from such diets. Haemostatic properties and serum lipoproteins were assayed, not only because of their suggestion by the literature, but for comparison to the study of fatty acid composition of erythrocytes.

## Materials and Methods

**i: Experimental Animals**

Twenty male and twenty female adolescent Cynomolgus monkeys (Macaca fascicularis) mean age 6.45 +/- 1.77 (mean +/- S.D.) years that had previously been used for testing poliomyelitis vaccine at Connaught Laboratories, Toronto, ON and used for testing zinc supplements in the Bureau of Nutritional Sciences, then rested for 4 months, were studied. They were randomized by a complete block design, on a weight and sex basis, into four dietary groups. Randomization was completed nine weeks prior to testing, to minimize any psychosociological stress and allow for a pilot study. All medication, including anti-inflammatories, steroids and glucocorticoids were contraindicated. The overall design of the study allowed sufficient time for two turnovers of the monkey erythrocytes.

The animals were housed in isolation in an animal care facility on a 12-hour (0700-1900 hr) light schedule at 24°C and 70% humidity. During the study, animal health and demeanour were recorded daily as a general check and by a detailed clinical examination each month. Health status was also ascertained by monitoring body weights weekly, throughout the study. All animals were in good to excellent health at the time of necropsy.

**ii: Diets**

The basal diet is shown in Table 1, dietary fat was the major test parameter. Monkeys in study group 1 (controls) received a lard (Canada Packers Ltd., Toronto, ON)/corn oil (Mazola, Best Foods, Montreal, P.Q.) blend, providing the majority of the polyunsaturated (PUFA) from the

(n-6) family of fatty acids and a very low content of (n-3) fatty acids. Group 2 received a lard/linseed (Maple Leaf Monarch Limited, Toronto, ON) oil blend, designed to provide a similar total PUFA content with equal amounts of (n-6) and (n-3). Similarly, groups 3 and 4 were provided with a lard/corn oil/menhaden oil (Zapata Haynie Corp., Reedville, VA). Diets 2, 3 and 4, all contained the same amount of PUFA and (n-6) fatty acids but differed in the source of (n-3) (Table 5). Linseed oil has a high alpha-linolenic acid (18:3, n-3) content, a short chain (n-3). The menhaden oil in diets 3 and 4 provided a rich source of (n-3) fatty acids, especially eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids, longer chain derivatives of the (n-3) family. Compared to other marine oils, menhaden has a low monoene content. Diet 4 differed from diet 3, by theoretically containing five times more alpha-tocopherol, a natural antioxidant (Table 4). However, when the alpha-tocopherol content of the unstripped dietary oils was considered, diet 4 experimentally had four times more alpha-tocopherol than diet 3 (or diets 1 and 2) (Table 2).

a) Special Considerations for Diet Formulations in Nonhuman Primates

There were some initial considerations involved in a dietary study of nonhuman primate models. Unlike other animal species, monkeys are selective eaters, thus the researcher must deliver a palatable diet in an acceptable format.

Nonhuman primates feed themselves with their hands. Diets presented in a meal form are not conducive to this kind of feeding behaviour. Because a moderate fat content was chosen (15%), commercial pelletized chows already containing 5% fat were unacceptable. The addition of fat

Table 1: Diet Composition

| Nutrient | % content (weight) | g per batch |
|---|---|---|
| Sucrose | 26.45 | 3041.8 |
| Corn Starch | 21.07 | 2423.2 |
| Casein | 21.43 | 2464.3 |
| Alphacel | 10.00 | 1150.0 |
| Mineral Mix (Teklad 79033) | 4.75 | 546.3 |
| Vitamin Mix (Teklad 85518) or (Teklad 85519)[1] | 0.50 | 57.5 |
| D,L-Methionine[2] | 0.50 | 57.5 |
| Choline Chloride[3] | 0.30 | 34.5 |
| Agar (2.5%)[4] | | 4.6 L |
| Orange Flavouring | | 51 mL |
| Fat[5] | 15.00 | 1725.0 |
| | | 11500.1 |

[1]Diet 4
[2]No. 9500, Sigma Chemical Co., St. Louis, Mo.
[3]No. 30200, Sigma Chemical Co., St. Louis, Mo.
[4]No. 160010, U.S.P., Sigma Chemical Co., St. Louis, Mo.

| [5]Fats: | | | |
|---|---|---|---|
| Diet 1 | Lard | 63.75 % | 1099.7 g |
| | Corn Oil | 36.25 % | 625.3 g |
| Diet 2 | Lard | 66.00 % | 1138.5 g |
| | Linseed Oil | 34.00 % | 586.5 g |
| Diet 3 & Diet 4 | Lard | 15.60 % | 269.1 g |
| | Corn Oil | 16.10 % | 277.7 g |
| | Menhaden Oil | 68.30 % | 1178.2 g |

Table 2: Alpha- and Gamma-Tocopherol Content of Diets (ug/g diet)[6]

| Diet | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| alpha-Tocopherol | 55.4 | 46.8 | 48.6 | 213.3 |
| gamma-Tocopherol | 46.6 | 7.4 | 6.7 | 5.1 |

[6]Evaluated at three intervals (in triplicate) during study

Table 3: Ausman-Hayes Mineral Mix (Teklad 79033)[1,2]

| | | g/kg |
|---|---|---|
| Potassium phosphate, dibasic | $K_2HPO_4$ | 327.908 |
| Calcium carbonate | $CaCO_3$ | 290.485 |
| Sodium chloride | NaCl | 162.366 |
| Magnesium sulfate | $MgSO_4-7H_2O$ | 98.732 |
| Calcium phosphate, dibasic | $CaHPO_4-2H_2O$ | 72.597 |
| Magnesium oxide | MgO | 32.040 |
| Ferric citrate | (16.7% Fe) | 13.297 |
| Manganese sulphate | $MnSO_4-H_2O$ | 1.221 |
| Zinc chloride | $ZnCl_2$ | 0.915 |
| Cupric sulphate | $CuSO_4-5H_2O$ | 0.290 |
| Potassium iodide | KI | 0.077 |
| Chromium acetate | $Cr(C_2H_3O_2)_3$ | 0.044 |
| Sodium fluoride | NaF | 0.023 |
| Sodium selenite | $Na_2SeO_3$ | 0.004 |

[1]designated for use at the rate of 4.75% in a nonhuman primate diet
[2]Teklad, Division of Harlan Sprague-Dawley Inc., Madison, Wi.

Table 4: Modified Hayes Vitamin Mix (Teklad 85518 or 85519)[3]

| | g/kg |
|---|---|
| Ascorbic acid, coated | 124.291 |
| i-Inositol | 100.984 |
| Taurine | 50.492 |
| D,L-alpha-Tocopheryl acetate (500 IU/g) | 20.197[4] |
| Niacinamide | 8.079 |
| Dry Vitamin A palmitate (500,000 IU/g) | 5.049 |
| Calcium pantothenate ($B_5$) | 3.297 |
| Riboflavin ($B_2$) | 1.616 |
| Folate | 0.809 |
| Thiamin HCl ($B_1$) | 0.808 |
| Dry Vitamin $D_3$ (500,000 IU/g) | 0.505 |
| Pyridoxine HCl ($B_6$) | 0.500 |
| Menadione | 0.101 |
| Biotin | 0.040 |
| Cobalamin ($B_{12}$) | 0.030 |
| Dextrin, white, technical | 633.203 |

[3]designated for use at the rate of 0.5% in a nonhuman primate diet
[4]Diet 4: D,L-alpha-Tocopheryl acetate added at the 5-fold level, 100.984 g/kg, at the expense of dextrin (TD 85519)

Table 5: Fatty Acid Composition of Diets (Area %)[1]

| Fatty Acid | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|
| C12:0 | 0.7 | 1.1 | 2.1 | 0.4 |
| C14:0 | 2.1 | 2.3 | 12.9 | 12.9 |
| C14:1 | | | 0.9 | 0.7 |
| C14:1 isomers | | | 0.2 | 0.5 |
| C16:0 | 28.6 | 27.5 | 25.1 | 25.8 |
| C16:1 (n-7) | 2.5 | 2.6 | 10.5 | 10.8 |
| C16:1 isomers | | | 0.5 | 0.3 |
| C17:0 | 0.3 | 0.3 | 0.1 | 0.2 |
| C17:1 | 0.3 | 0.3 | 1.4 | 1.5 |
| C17:1 isomers | | | 0.5 | 0.5 |
| C18:0 | 7.7 | 8.4 | 3.6 | 3.7 |
| C18:1 (n-9) | 32.1 | 32.4 | 16.4 | 16.9 |
| C18:1 isomers | 0.2 | 0.2 | 1.5 | 1.5 |
| C18:2 (n-6) | 24.7 | 11.8 | 10.6 | 10.8 |
| C18:2 isomers | | | 1.6 | 1.5 |
| C18:3 (n-3) | 0.4 | 13.0 | 0.9 | 0.8 |
| C18:3 (n-6) | | | 0.1 | 0.1 |
| C18:4 (n-3) | | | 1.8 | 1.9 |
| C19:0 | | | 0.1 | 0.1 |
| C20:0 | 0.3 | 0.1 | | |
| C20:1 (n-9) | 0.2 | | 0.4 | 0.5 |
| C20:1 isomers | | | 0.1 | 0.2 |
| C20:2 (n-6) | 0.1 | | 0.2 | 0.3 |
| C20:4 (n-6) | | | | |
| C20:4 (n-3) | | | 0.3 | 0.4 |
| C20:5 (n-3) | | | 4.7 | 4.6 |
| C22:0 | | | 0.2 | |
| C22:1 (n-11) | | | 0.3 | |
| C22:1 isomers | | | | 0.5 |
| C22:4 (n-6) | | | | |
| C22:5 (n-6) | | | | |
| C22:5 (n-3) | | | 0.4 | 0.2 |
| C22:6 (n-3) | | | 2.8 | 2.4 |
| ∑(n-6) | 24.8 | 11.8 | 10.8 | 11.2 |
| ∑(n-3) | 0.4 | 13.0 | 10.9 | 10.2 |
| ∑(saturates) | 39.7 | 39.7 | 43.9 | 43.1 |
| ∑(monounsaturates) | 35.2 | 35.5 | 32.8 | 35.5 |
| ∑(polyunsaturates) | 25.1 | 24.8 | 23.2 | 21.3 |
| ∑(n-6)/∑(n-3) | 64.0 | 0.9 | 1.0 | 1.1 |

[1]mean composition as determined at three intervals during study

would dilute the concentration of the other nutrients as well as decrease consumption by increasing the energy level. Although pelletized diets are the most palatable physical form, their manufacture allows for only limited amount of fat. Additionally, the application of heat or pressure to a highly polyunsaturated oil is questionable in a dietary study. Thus, in this study, a novel form of diet, a dough formulation was utilized. The monkeys were exposed to a control diet prior to the study so that the monkeys best adapted to eating dough balls were selected from a larger colony. The animals were fed 160 g/day (660 kcal) which was in excess of their daily requirement of 120 kcal/kg body weight (Nicolosi and Hunt, 1979) to compensate for the addition of agar to the basal diet (in a ratio of 2:5) and for the large amount of diets wasted in feeding. This wastage also rendered the evaluation of intake infeasible but body weights were monitored. The animal cages were placed in well-spaced banks to avoid cross-contamination of the study diets.

To minimize oxidation, the diets were made weekly, were mixed for only a short duration and were packaged in daily portions. These portions were stored in polyethylene bags at 4°C; there were no problems with mold. The diet was presented to the animals twice daily as two 40 g balls at 0830 and 1430 hours. Although the majority of the animals ate the diet upon receipt, any remainders were discarded.

Choice of Nutrients

The Newberne-Hayes Semipurified Primate diet (1979) was adopted, with some modifications, since it was specific to the needs of juvenile and adult Cynomolgus monkeys.

Protein

Casein (91%) protein was selected to provide the source of protein in the study. It was provided at a level to deliver protein at 19.5% by weight, although Newberne and Hayes (1979) suggested that 12-16% protein is adequate for this species. Among protein sources: lactalbumin, soy protein isolate and casein; casein has the advantages of being well-tolerated and of high biological value. D,L-methionine was supplemented to the level of 0.5%, since it is a limiting amino acid in casein (Sarwar, Peace and Botting, 1985).

Carbohydrate

Sucrose, dextrose or dextrin/starch are all potential sources of carbohydrate. It is suggested that this species fares well on a blend of sucrose and dextrin. Increasing the sucrose content increases palatability, while increasing dextrin does not. The level of corn starch used was slightly decreased to facilitate more protein.

Fibre

Other dietary guidelines suggested little to no fibre while Newberne and Hayes (1979) suggested fibre be added at the level of 5-10%. The level was adjusted upon examination of a sample of stool.

Vitamins

Vitamins were added at the level suggested by Newberne and Hayes (1979). Pantothenate and pyridoxine were also added. Coated ascorbic acid was selected to minimize its oxidation.

Minerals

Several guidelines suggested Hegsted IV Salts as the mineral mix. According to Newberne and Hayes (1979), it is low or deficient in

magnesium, zinc, selenium, chromium and fluoride, while excessive in iron and iodide. The Hawk-Oser (1954) mix suggested in the NRC guidelines (1978) lacks copper, selenium, fluoride and cobalt. More recent versions of the Ausman-Hayes mineral mix have increased the selenium content and decreased the fluoride content, also adopted by this study.

Choline Chloride

Choline chloride provided the source of choline in the diet. This ingredient was mixed directly into the diet since it has the potential to oxidize B-vitamins if stored in a vitamin pre-mix.

Agar

Agar served as a binding agent in the diet and reduced surface exposure to air. Carrageenan cannot be used in this instance since it is associated with gastrointestinal lesions in nonhuman primate studies (Nicolosi and Hunt, 1979). The agar content was adjusted from 80.0 ml of 1.25% (constituting 9.2 L per batch) to 4.6 L of 2.5%, this measure was taken to improve the consistency of the diet.

Flavouring

The monkeys preferred an orange flavouring to the suggested banana flavouring. In their natural environment, these animals do not feed on bananas. Flavouring, overall, was found to be beneficial in masking the pervasive odors of the dietary oils. In preliminary studies, 51 mL of flavouring per batch was ascertained to be optimal.

Fats

In this species, an acceptable fat content ranges from 20-40% of the caloric intake (Newberne and Hayes, 1979). Essential fatty acids must be provided at a minimum of 0.4 en%.

For this study, the fatty acid composition of each dietary oil was determined upon receipt. Separate containers were pooled into 50 L drums to provide homogeneity. Oils were stored at -40°C, under nitrogen gas. Blends were stored up to one week, in amber 4 L bottles at 0°C, under nitrogen.

Diet Acceptability

Early exposure to the diet indicated that the animals were becoming dehydrated, with a corresponding weight loss. As a remedy, 500 mL of fruit juice (apple) was given along with 1000 mL of water daily. Fruit juice and a carrot or half-apple enhanced the acceptability of the diet.

**iii: Sample Collection**

On weeks 0,1,4,6,9,12,15, 10 mL of blood was drawn into a vacutainer tube containing 1/10 volume of 3.8% sodium citrate (Becton-Dickinson, Oakville, ON). Sodium citrate was chosen as the anticoagulant, so as not to interfere with the subsequent thiobarbituric acid assays. To facilitate atraumatic sampling, the fasting animals were immobilized with 5 mg/kg Rogarsetic (ketamine hydrochloride, Bristol Laboratories, Syracuse, N.Y.). The blood samples were analyzed on a Coulter Counter (Model S Plus IV, Mississauga, ON) for haematological profiles. Aliquots of whole blood were taken for the thiobarbituric acid (TBA) assay, while the remaining bulk of the blood was fractionated. Platelet-rich and platelet-poor fractions were isolated, washed and stored at -70°C. Haematological profiles of purified erythrocytes were also obtained. Then 100 uL aliquots for superoxide dismutase (SOD) and glutathione

peroxidase (GSH-Px) assays along with the remaining packed cells were frozen at -70°C, under nitrogen, until further analysis.

A known mass of red blood cells was extracted and stored as a stock solution at 4°C. A portion of the stock solution was subjected to methylation and subsequent gas chromatographic analysis. At weeks 1,3,6,9 and 12, bleeding time and bleeding time blood volume assays were completed. Reliable blood pressure data, however, could not be obtained. On weeks 14 and 15 (99 to 108 calendar days) the animals were euthanized in groups of four and five. The euthanasia procedure consisted of the administration of Rogarsetic followed by intracardiac puncture and exsanguination. Samples of ventricular blood were retained for lipoprotein analyses. The bodies were perfused with citrated saline. Grossly visible fat deposits, vascular integrity and lesions were investigated. Several tissues: median lobe of liver, auricles, ventricles, right kidney, lung, gastrocnemial muscle, epicardial and perirenal adipose, testes/ovaries, adrenals, spleen, mesenteric lymph nodes, thymus, retinas and frontal cortex were dissected, weighed and placed in dry ice and then stored at -70°C for future reference.

There are potential hazards that may arise with the use of nonhuman primates in biomedical research. These animals may harbour infectious microorganisms and parasites that can be pathogenic for man; some are especially hazardous: Herpesvirus simiae, rabies, hepatitis A and Marburg viruses. The possibility of interspecies disease transmission can compromise both the integrity of the experiments and the health of the persons exposed to the animals. To minimize the inherent biohazards in this experiment, standard operating procedures involving the isolation of

the animals, protective clothing for the researchers and attention to laboratory waste disposal had to be maintained through the experiment, in its entirety.

**iv: Blood Fractionation**

The purpose of the blood fractionation procedure was to purify the platelets, plasma, leukocytes and RBC components for separate analyses. Whole citrated blood was mixed thoroughly (Haematology Mixer, Fisher Scientific, Nepean, ON). Platelet-rich (PRP) and platelet-poor (PPP) plasma fractions were collected, as suggested by Ahmed and Holub (1984) but were isolated by the method of Roshanai and Sanders (1985). PRP was isolated by centrifugation (IEC Centrifuge HN-SII, Fisher Scientific, Nepean, ON) at 120 g x 15 minutes at room temperature, a procedure allowing for the maximal harvesting of platelets in the supernatant (Woods, Dennehy and Clarke, 1976). This was followed by a second spin at 1500 g x 15 minutes to obtain the PPP. Both fractions were siphoned into disposable transfer pipettes (Fisher Scientific, 13-711-8) and stored in cryogenic vials (Sarstedt, Montreal, P.Q.) at -70°C, under nitrogen gas. The resultant platelet concentration was determined for both PRP and PPP fractions. RBC and WBC counts were determined likewise for quality control. The results of WBC were discarded due to low cell counts determined in whole blood ($9.45 \times 10^3$ +/- $2.55 \times 10^3$, mean +/- S.D., n=40). The remaining RBC were washed twice more to remove any residual contaminants in two volumes ice-cold isotonic saline (8.9 g/L). Aliquots were removed for enzyme and haematological assays. Analysis of this

fraction showed a mean WBC count of $4.58x10^3$ +/- $0.587x10^3$(4) and a mean platelet count of $51.4x10^3$ +/- $23.4x10^3$(4). It would appear unlikely that the cells contributed any appreciable contamination, since the mean RBC count was $6.83x10^6$ +/- $0.135x10^6$(4). The packed cells (approximately 3.0 mL) were also transferred to cryogenic vials and stored at -70°C, under $N_2$ (g). The results of lipid analyses were expressed as ug fatty acid per $10^6$ RBC.

**v: Erythrocyte Extraction**

One of the most versatile and effective lipid extraction procedures is the Bligh and Dyer (1959), a simplified version of the classical Folch, Lees and Stanley (1957) procedure (Kates, 1972).

The lipids were extracted from the erythrocytes by a modified Bligh and Dyer method. Approximately 3.0 g of the unthawed RBCs were accurately weighed (Mettler AE 100 Analytical Balance, Fisher Scientific, Nepean, ON) in a 60 mL separatory funnel, sufficient water was added (erythrocytes contain 65% water) to give a final volume of 6.0 mL. Then 7.5 mL chloroform, containing 50 mg/L BHT[5] (Sanders et al., 1981) and 15.0 mL methanol was added to the separatory funnel and thoroughly shaken, to produce a monophasic solution. The addition of the methanol lysed the cells, facilitating lipid extraction. Another 7.5 mL of chloroform were added and the admixture was shaken. Upon the addition of 7.5 mL water, the separatory funnel was inverted, rather than shaken, ten

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[4]mean cell count +/- standard deviation (weeks 0, 1, 4, 9, 15) in 100 uL packed RBC, diluted 1:1 with isotonic saline, n=200.

[5]Butylated Hydroxytoluene, Sigma Chemical Co., St. Louis, Mo.

times. To separate the phases the separatory funnels were then stored overnight at 4°C. Then 13.0 mL of the lower chloroform phase was transferred to a 125 mL round-bottom flask, with the addition of 1.0 mL absolute alcohol to aid in the removal of any potential traces of water (Christie, 1982). The chloroform phase was then concentrated, to dryness, on a rotary evaporator (Buchi Rotavapor R-110, Switzerland), under $N_2$ (g). The dried sample was then immediately reconstituted with chloroform and quantitatively transferred to a 10 mL volumetric flask which contained 0.2 mL methanol. The stock solution was stored at 4°C.

**vi: Methanolysis**

To determine the fatty acid composition of the erythrocytes the volatile methyl esters were then derived (Beare-Rogers, Gray, Nera and Levin, 1979). In a combination hydrolysis-extraction flask (Kates, 1972)[6] 0.75 mL 0.1% hydroquinone (AnalaR, BDH Chemicals, Toronto, ON), an antioxidant, was added prior to the addition of 3.0 mL stock solution. Samples were evaporated under direct flow of $N_2$ (g) on the 'N-Evap' apparatus (Model 10, Organomation Associates, Shrewsbury, Mass.). Then 2.0 mL 0.5 N NaOH (AnalaR, BDH Chemicals, Poole, Eng.) in methanol was added to hydrolyze the fatty acyl moieties. The samples were refluxed 5.0 minutes and cooled for 1 minute. The formation of methyl esters was catalyzed by the addition of 2.0 mL 14% w/v $BF_3$-methanol[7] (Morrison and Smith, 1964) and refluxed for 2.0 minutes. At this point, 13.6 uL of

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[6]Köntes Co. Ltd., Division of Mandel Scientific Co. Ltd., Rockwood, ON

[7]Chromatographic Specialties, Brockville, ON

2.000 mg/mL methyl ester of nonadecane (Sigma Chemical Co., St. Louis, Mo.) was added as the internal standard. Quantitative removal of the methyl esters was completed upon washing thrice with 5.0 mL hexane. Samples were then kept in tapered ground-glass stoppered volumetric tubes at 4°C, for gas chromatographic analysis, usually within a day.

**vii: Gas Chromatography**

The gas chromatographic analysis for this study was performed on hexane-dissolved samples using a Varian Vista 6000 (Varian Canada Inc., Ottawa, ON) chromatograph equipped with flame ionization detectors and a Supelcowax 10M fused silica capillary column, 30 m x 0.32 mm I.D., film thickness 0.25 um (Supelco Inc., Bellefonte, PA.) with hydrogen as the carrier gas. The detector temperature was 275°C; the injector temperature was 250°C; the oven temperature was initially 130°C for 12 minutes, then increased at a rate of 2°C/min. to a final temperature of 180°C, which was maintained for 18 minutes. The programmed run took 55 minutes.

Temperature programming was necessitated by the range of fatty acids, allowing for the passage of shorter chain fatty acids, followed by a steady temperature increment to allow a reasonable analysis time and to improve resolution of the longer chains. Peaks were identified by comparison with retention times of known fatty acid methyl esters kits: GLC Reference Mixture[8], GLC-60[9], Qualmix 4-705[9], L-209[10], K-103[10],

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[8]NuChek Prep, Elysian, Minn.

[9]Supelco Inc., Bellefonte, PA.

[10]Applied Science, Division of Milton Roy Co., State College, PA.

containing methyl esters of 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 20:4, 20:5, 22:6; additionally, individual methyl esters of 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0 and 24:0[9] were available for use as primary standards. PUFA-1, PUFA-2 and rapeseed oil mixture[9] were utilized as secondary reference standards, providing a spectrum of (n-6) and (n-3) polyenoic fatty acids. The identification of fatty acid peaks was compared to the profile derived from SP-2330 30M capillary column[9] operated isothermally at 190°C. The above procedure was routinely carried out on blank samples to exclude the presence of any artifactual peaks and was punctuated by checks of retention times against standards upon elevation of oven temperature overnight. Calibration was also facilitated by the use of methyl ester of 19:0 as the internal standard. Its retention time corresponded to the midpoint of the analysis and it did not co-elute with any other fatty acid methyl ester. Hydroquinone, utilized in the methanolysis procedure, co-eluted with the hexane void volume hence it had no impact on the chromatograms.

Peak areas were calculated by means of an electronic integrator relative to the internal standard. Results are expressed in absolute terms of ug fatty acid per $10^6$ RBC.

**viii: SOD Activity in Erythrocytes**

Superoxide dismutase (SOD) was analyzed by an indirect method whereby the generation of superoxide radical ($^{\bullet}O_2$) by xanthine-xanthine oxidase was coupled to the reduction of cytochrome C. A decreased

reduction of cytochrome C, upon the SOD-catalyzed disproportionation of $^{\bullet}O_2$, was measured spectrophotometrically (L'Abbe and Fischer, 1986).

Reagents and procedure adhered to the method of L'Abbe and Fischer (1986) employing an Abbott ABA-200 series II bichromatic discrete analyzer (Abbott Laboratories Ltd., Mississauga, ON) equipped with a 415/450 nm filter. Sodium carbonate, sodium hydrogen carbonate and EDTA, disodium salt, were purchased as AnalaR analytical reagents, from BDH Chemicals, Poole, Eng., ferricytochrome C, SOD and xanthine oxidase from Sigma Chemical Co., St. Louis, Mo., xanthine from Fisher Scientific Co., Fair Lawn, N.J., according to the procedural specifications.

To 100 uL packed RBC from weeks 0,4,9 and 15, 900 uL of distilled water was added. Then 500 uL of the lysed cells was extracted with 200 uL chloroform/ethanol (0.6:1.0), mixed with a vortex mixer and centrifuged at 15,600 x g for 3 minutes, in an Autocrit II centrifuge (Fisher Scientific, Nepean, ON). The resultant supernatant was decanted and employed in the analysis of Cu,Zn-SOD.

Six standards of fresh SOD, ranging in activity from 27.0 to 135 U/mL, representing 78 to 34% of the initial reaction rate, were run in parallel with the samples, for the construction of a batch standard curve. SOD activity in the erythrocytes was determined from the equation of the standard curve. This value was converted to units of activity/mL packed RBC. Results were then expressed in terms of units/mg haemoglobin x $10^{-1}$.

**ix: Glutathione Peroxidase Activity in Erythrocytes**

The method determined the total glutathione peroxidase (GSH-Px)

activity in erythrocytes as outlined by Levander, de Loach, Morris and Moser (1983) based on the work of Paglia and Valentine (1967). GSH-Px produces oxidized glutathione (GSSG) in red cells. The formation of GSSG was measured by the glutathione reductase-catalyzed reaction with the coupled conversion of NADPH to $NADP^+$, which was monitored spectrophotometrically. In stoichiometric terms, one mole of NADPH is oxidized for each mole (t-butyl) hydroperoxide reduced. Adaptations for analysis on the Abbott ABA-200 series II bichromatic discrete analyzer (Abbott Laboratories Ltd., Mississauga, ON) were made by L'Abbe (unpublished).

For this assay, 200 uL of the RBC-lysate prepared in the SOD assay was added to an equal volume of quadruple-strength Drabkin's reagent (SDR, BDH Chemicals, Poole, Eng.), to convert haemoglobin to the stable cyanmethaemoglobin form. This inhibited the activities of catalase, methaemoglobin reductase and NADPH-dependent diaphorase, which also act on peroxides in erythrocytes, while not altering GSH-Px activity (Paglia and Valentine, 1967). Subsequent addition of azide, in conjunction with the cyanide and for the same purpose, has been found beneficial (Paglia and Valentine, 1967). To a reagent mix containing 150 mL 1.0 M $KH_2PO_4$[11]/$K_2HPO_4$[12], 25 mL 0.2 M EDTA[13], disodium salt, 200 mL 0.1% sodium hydrogen carbonate[13] and 50 mL 10 mM sodium azide[14] per litre, 61.5 mg GSH (No. G-4251)[14], 44 uL GR (No. G-4751, Type III, highly purified from Baker's Yeast)[14] and 20 mg NADPH (B-nicotinamide adenine dinucleotide,

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[11]certified A.C.S., Fisher Scientific, Nepean, ON

[12]Aldrich Chemical Co. Inc., Milwaukee, Wi.

[13]AnalaR, BDH Chemical Ltd., Poole, Eng.

[14]Sigma Chemical Co., St.Louis, Mi.

reduced form, No. 6505, Type III)[14] was added daily. The enzymatic reaction was initiated by the addition of 1.2 mM t-butyl hydroperoxide (No. B-2633)[14]. The decrease in absorbance at 340 nm, upon conversion of NADPH to $NADP^+$, was followed in five minute intervals, for 15 minutes.

The nonenzymatic oxidation of GSH was determined by the simultaneous assay of the system substituting the hemolysate with distilled water, in quadruplicate. The reaction rate of the latter system was subtracted from that of the former, to determine enzymatic activity, in triplicate. The results, in units GSH-Px/L assay mixture, were corrected for assay dilutions and converted to units GSH-Px per mg haemoglobin.

**x: Thiobarbituric Acid Assay**

Malondialdehyde (MDA) is an end product of *in vivo* lipid peroxidation and is often used as an indication of this process. The thiobarbituric acid (TBA) method for the assay of MDA is the most appropriate because of its sensitivity (Placer, Cushman and Connor-Johnson, 1966; Yagi, 1984). One molecule of MDA reacts with two molecules of TBA, with the elimination of water, to yield a red chromagen with an absorption maximum at 532-535 nm. Although MDA has been identified amongst the products of oxidative decomposition of amino acids, complex carbohydrates and pentoses/hexoses, its major source is the secondary fragmentation products of PUFA peroxides derived from fatty acids with three or more double bonds (Bird and Draper, 1984). MDA is also a by-product of cyclooxygenase and thromboxane synthetase activities (Smith, Ingerman and Silver, 1976; Tomita, Umegaki, Hayashi, 1983). Autoxidation products consist of aldehydes, including MDA, as well as

hydroperoxy alkenyls, hydroperoxy epoxides and polymers; hence this assay should be considered as one for thiobarbituric acid reactive substances 'TBARS' (Yoshikawa, Tanaka and Kondo, 1985).

TBARS were assayed by several methods: Placer et al. (1966); Stocks and Dormandy (1971); Ohkawa, Ohishi and Yagi (1979); Asakawa and Matsushita (1980) and Kanazawa, Kanazawa and Natake (1985); however, the method of Buege and Aust (1978) with modifications was chosen. In screw-cap test tubes, 100 uL of whole citrated blood (Smith et al., 1976) was combined with 300 uL concentrated Drabkin's solution (10 mg/mL cyanide) added to inhibit catalase activity (Stocks and Dormandy, 1971) and to complex with haemoglobin (Thorngren et al., 1983c) resulting in an absorbance maximum 418 nm. Then 2.00 mL stock TCA[15]-TBA[16]-HCl was added and mixed thoroughly, upon saturation with nitrogen. The use of nitrogen rather than BHT has been found more effective at inhibiting concomitant autoxidation of lipids in vitro while not influencing the formation of the red chromagen (Kosugi and Kikugawa, 1985). The mixture was heated for 15 minutes at 100°C, the reaction was terminated by plunging into an ice-water bath. The flocculent precipitate of subcellular particles was removed by centrifugation at 3000 rpm for 15 minutes. The absorbance of the sample was determined (Cary Model 2200 spectrophotometer, Varian Canada Inc., Ottawa, ON) at the absorbance maximum 532 nm against blanks containing all reagents minus RBC. Isosmotic phosphate buffer

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[15]Fisher Scientific, Nepean, ON.

[16]2-thiobarbituric acid (4,6-dihydroxy-2-thiopyrimidine), Sigma Chemical Co., St. Louis, Mo.

($KH_2PO_4$[17]/$K_2HPO_4$[15]) was the solvent or diluent of the various reagents as well as the blanks (Stocks and Dormandy, 1971).

To determine MDA content, a stock solution of 10 mM MDA was made by hydrolyzing 200 mg TMP (1,1,3,3-tetramethoxypropane)[18] in 100 mL 1% $H_2SO_4$. The stock solution was diluted with 1% $H_2SO_4$, absorbance was ascertained at 245 nm (extinction coefficient= 13700. $M^{-1}cm^{-1}$) and corrected to $1.678 \times 10^{-5}$ M, on the day of analysis (Smith et al., 1976). Aliquots of 0.020-0.100 mL of standard were run in parallel to the triplicate samples throughout the procedure. The results, as determined from the linear equation of the batch standard curves, are reported in absolute terms of mol$\times 10^{-9}$ MDA/$10^6$ RBC.

**xi: BT and BTBV Assays**

Bleeding time (BT) is defined as the time interval between the making of a small incision and the moment when bleeding stops. It is generally considered as the combined haemostatic response of small vessels, capillaries, arterioles and venules, severed simultaneously under standardized conditions (Thorngren, Shafi and Born, 1983b,c,d).

The BT assay was conducted by the method of Thorngren et al. (1983b) utilizing the 'Simplate II' device, a sterile disposable template system with spring-loaded blades designed to make uniform incisions (General Diagnostics, Division of Warner-Lambert Company, Toronto, ON). According to the method, a constant pressure of 40 mmHg was applied to the upper

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[17]Aldrich Chemical Co. Inc., Milwaukee, Wi.

[18]malonaldehyde bis (dimethyl acetal), Aldrich Chemical Co. Inc., Milwaukee, Wi.

arm with a sphygmomanometer. In an area free of vascularization, distal to the cubital fossa, two transverse incisions were made 5 mm long and 1 mm deep. In this application, the monkey's leg was found preferable. The blood emerging from the incisions was removed quantitatively with a Whatman #1 filter, ensuring not to disturb clot formation, until bleeding stopped. The results are expressed in minutes. Generally, the leg incisions healed within 24 hours.

Bleeding time blood volumes (BTBV) were measured by eluting the blood from the filters into 20.0 mL Drabkin's reagent (BDH Chemicals, Poole, Eng.) on a cell mixer for five hours. For each eluate, the haemoglobin concentration was determined spectrophotometrically (Cary Model 2200, Varian Canada Inc., Ottawa, ON) at the absorbance maxima of 418 nm. Absorbance readings, in triplicate, were converted to their corresponding blood volumes, obtained from individual standard curves constructed by using 25, 35, 45 and 55 uL of each animal's whole citrated blood[19] in 10.0 mL Drabkin's reagent, $r>0.99$. All samples had to be diluted further to 10%. The results of the BTBV assay are expressed in uL whole blood.

**xii: Serum Cholesterol**

Intracardial blood samples obtained at necropsy were centrifuged at 1500 x g for 30 minutes (Beckman Model J21C, Beckman Instruments Canada Inc., Toronto, ON). HDL-cholesterol was isolated from serum by re-centrifugation at 12900 x g for 5 minutes (MSE Micro Centaur, Johns Scientific Inc., Nepean, ON). Serum fractions were analyzed for total

[19] corrected for the dilution by citrate

cholesterol and HDL-cholesterol using the Abbott A-Gent Cholesterol test. Likewise, serum triglycerides were analyzed with the Abbott A-Gent Triglycerides test. Assays were completed following the ABA-100 procedures, on the Abbott ABA-200 Bichromatic Analyzer (Abbott Laboratories, Diagnostics Division, Mississauga, ON). Results were expressed in mg/dL.

**xiii: Statistical Analyses**

The data were analyzed for analysis of variance (ANOVA) with repeated measures (refer to Appendix). The data were grouped according to dietary treatment, to sex and to time interval; all interactions of these grouping factors were also analyzed for. In some instances, sex and the interaction of sex by group were statistically significant; but due to low cell sizes pertaining to these factors (n=5), further statistical evaluation was curtailed. Indication was provided, however, that such significances existed. The ANOVA table also presented the orthogonal decomposition of the data into polynomial components. The consequential trends in the data, in terms of linear, quadratic, cubic and more complex forms of mathematical equations were provided in the results section.

Collection of haematological values during the study provided several parameters for study: leukocyte counts,, erythrocyte counts, haemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and platelet counts. Preliminary statistical evaluation indicated that there were no statistically significant differences. Platelet counts were further

analyzed since they were the only cells to demonstrate a dietary trend and secondly, platelets have been shown in the literature to be manipulated by similar studies.

The erythrocytes were harvested and analyzed in both the sixth and the twelfth week of study. The gas chromatograph column had deteriorated by the time the week 12 samples were to be analyzed. An incidence of hemolysis in week 6 yielded incomplete records for 6/40 of the animals; although there were statistical alternatives for missing data, this time interval was also discarded from fatty acid analyses. A review of the results indicated that dietary manipulations were apparent before this time point. The BMDP program utilizes only cases with complete data records, for all variables. Four blood specimens were lost in the fractionation step, during one week of the study; hence the complete set of data for these animals was excluded in the computations. The net number of replicates was 36, based on data collected from weeks 0,1,4,9 and 15.

There were 39 fatty acids monitored throughout the study; however only (n-6) and (n-3) and other fatty acids exceeding 0.1 ug/$10^6$ RBC were presented. Minor fatty acid components contributed to summations of their family (i.e. total monounsaturates, total saturates).

Duncan's New Multiple Range test was employed as the post hoc test (refer to Appendix); this test was applied at the 5% level of significance for all assays, excepting the fatty acid composition analyses, where the 1% level of significance was applied. A more stringent test was necessitated in this instance, due to the vast number of significances, compounded by interactions. The 1% level of

significance was therefore intended to identify the most salient significant differences, as a consequence, the biological application of the findings may be diminished. Some of the other analyses approached the 5% level of significance, the respective tail probabilities were provided, to indicate possible trends in the data. These trends could very well become significantly different, given a larger cell size or an increased sampling.

The Duncan's table of Significant Studentized Ranges only allowed for the comparison of up to 20 means. There were occurrences in its application, where the maximum and minimum means appeared to be significantly different; however, the comparison of the 40 means involved was not permitted by this statistical test. These means therefore were presented as not being significantly different.

Duncan's Multiple Range test indicated significant differences of all interactions. For simplicity, only significances among groups were compared at the same time interval and only significances of time were compared for the same group.

The statistical significant differences along with their corresponding mean and standard error of means (S.E.M.s) were presented in the tables in the 'Results' section for each fatty acid. The plots present the results of each dietary group; the scale of the plots was standardized for ease of comparison. Except for Figure 1, all plots are provided in the 'Appendix'.

# Results

## i: Effect of Dietary (n-3) on Fatty Acid Composition of Erythrocytes

a) Effect on (n-6) Fatty Acids

Individual fatty acids from monkey erythrocytes were determined for each group and each time as shown in Figures 2 to 17 (refer to Appendix). Tables 6 to 10 display each (n-6) fatty acid individually for group comparison; Figures 2 to 9 portray the (n-6) family of fatty acids for each group of males and of females.

In Table 6, the levels of linoleic acid (18:2, n-6) showed dietary differences which became apparent within the first week of study. Groups 1 and 2 demonstrated somewhat elevated levels compared to groups 3 and 4. By week 9, groups 1 and 2 exceeded groups 3 and 4 by at least two-fold. At the end of the study, males fed diet 1 had a significantly elevated LA content compared to those fed diet 2, both of which were significantly higher than monkeys fed diets 3 and 4.

Males in group 1 and males and females in groups 3 and 4 indicated a time effect. Males in group 1 (Figure 2) had achieved maximal linoleate content on the 15th week of study, a level which was significantly greater than at all previous time intervals. Females, while not significantly different from their male counterparts nor any other dietary group by the end of the study (Figure 3), failed to demonstrate dietary enrichment of 18:2 (n-6).

Monkeys fed diets 3 and 4, contrary to those fed diet 1, exhibited an attenuation of LA content. Both males and females had maximal linoleate content at zero-time (Figures 6 to 9), with a subsequent depletion, which became statistically significant within the first to

## Erythrocyte Fatty Acid Composition

Tables 6 to 10 represent individual (n-6) fatty acyl components of Cynomolgus monkey erythrocytes obtained by gas chromatographic analysis. Diet 1 contained lard and corn oil (high linoleic acid), Diet 2 contained lard and linseed oil (high linolenic acid) and Diets 3 and 4 contained lard, corn oil and menhaden oil (high long chain n-3). All diets were isocaloric and provided equal total polyunsaturate contents; however, Diet 1 provided (n-6), Diet 2 equal (n-6) to (n-3), Diets 3 and 4 equal (n-6) to longer chain (n-3) fatty acids, as the source of polyunsaturates. Diet 4 differed from Diet 3 by four-fold enrichment of alpha-tocopherol.

Table 6: Results of Linoleic Acid (18:2, n-6) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing same superscripts are significantly different.

Significant orthogonal components are 1, 2 and 4; linear and quadratic interactions with group; quadratic interaction with sex (p=0.0148); and time interaction with sex (p=0.0479).

Corresponding plots are provided in Figures 2 to 9.

Table 6:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 1.74 | 1.97 | 1.59 | 1.45 |
| | S.E.M. | 0.13 †b | 0.19 | 0.11 †a | 0.11 †a |
| 1 | | 1.51 *ade | 1.74 *a | 0.841 *b | 1.00 *be |
| | | 0.08 †b | 0.27 | 0.049 †b | 0.07 †ab |
| 4 | | 1.68 *a | 1.68 *a | 0.855 *b | 0.898 *b |
| | | 0.15 †b | 0.08 | 0.079 †b | 0.048 †b |
| 9 | | 1.52 *a | 1.77 *a | 0.664 *b | 0.658 *b |
| | | 0.08 †b | 0.12 | 0.019 †b | 0.051 †b |
| 15 | | 2.31 *a | 1.76 *b | 0.755 *c | 0.791 *c |
| | | 0.14 †a | 0.15 | 0.057 †b | 0.092 †b |
| Females | | | | | |
| 0 | | 1.89 | 1.61 | 1.38 | 1.57 |
| | | 0.08 | 0.12 | 0.06 †a | 0.17 †a |
| 1 | | 1.71 *a | 1.61 *ac | 1.04 *bcd | 1.09 *bcd |
| | | 0.12 | 0.08 | 0.06 †ab | 0.08 †ab |
| 4 | | 1.68 *a | 1.78 *ab | 0.782 *b | 1.12 *ab |
| | | 0.03 | 0.08 | 0.032 †b | 0.21 †ab |
| 9 | | 1.69 *a | 1.50 *a | 0.644 *b | 0.732 *b |
| | | 0.15 | 0.13 | 0.044 †b | 0.043 †b |
| 15 | | 1.87 *abc | 1.61 *b | 0.635 *c | 0.690 *c |
| | | 0.11 | 0.11 | 0.039 †b | 0.108 †b |

n = 36

fourth weeks. Females fed diet 4, showed a more gradual decline in LA levels; significance was attained by week 9.

By the end of the study, animals fed diet 1 exhibited a 32.6% increase in linoleate content; those fed diets 3 and 4 showed decreases to one-half of their original 18:2 (n-6) content. Both trends were in evidence early in the study.

Arachidonate (20:4, n-6), an important metabolite of linoleate, varied with time in males of group 1 and in females of groups 3 and 4. Males of group 1 (Figure 2) had arachidonate levels at the terminus that were maximal and significantly different from preceding weeks and that represented a 38% increment. Females of groups 3 and 4 displayed steady declines in arachidonate content (Figures 7 and 9). Females of group 3 (Table 7) showed a decrease from 1.26 to 0.443 ug AA/$10^6$ RBC, a change of -64.8% which became significant by week 9. Similarly, females of group 4 (Table 7) decreased from 1.50 to 0.411 ug AA/$10^6$ RBC, a change of -72.6% which became significant from week 4 values at week 15. Group differences of AA became apparent on the ninth week. By the end of the study, males of group 1 were significantly different from those of groups 3 and 4 but not from group 2. Females of group 2 were significantly different from those of groups 3 and 4 at week 9; this difference could not be identified at week 15. Thus, it appeared that erythrocyte AA was also affected by dietary manipulation and required 15 weeks to reach significance. It was also interesting to note that the magnitude of the depletion of 20:4 in those monkeys fed diets 3 and 4 was approximately twice that of the increase observed in males fed diet 1.

Table 7: Results of Arachidonic Acid (20:4, n-6) Analyses (ug/$10^6$ RBC).

Values represent group means +/- S.E.M. of fatty acid content at week 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing same superscripts are significantly different.

Significant orthogonal components are 1, 2, 4; linear and quadratic interactions with group; quadratic interaction with sex; group interaction with sex by orthogonal components 3 and 4; time interaction with sex (p=0.0275) and time interaction with sex by group (p=0.0259).

Corresponding plots are provided in Figures 2 to 9.

Table 7:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 1.32 | 1.55 | 1.63 | 1.41 |
| | S.E.M. | 0.06 †ab | 0.18 | 0.20 | 0.03 |
| 1 | | 0.949 | 1.27 | 0.931 | 0.948 |
| | | 0.089 †b | 0.19 | 0.048 | 0.060 |
| 4 | | 1.12 | 1.18 | 0.873 | 0.941 |
| | | 0.14 †b | 0.15 | 0.105 | 0.035 |
| 9 | | 1.00 *ab | 1.35 *ab | 0.509 *b | 0.451 *b |
| | | 0.07 †b | 0.11 | 0.031 | 0.027 |
| 15 | | 1.82 *a | 1.40 *ac | 0.480 *bc | 0.480 *bc |
| | | 0.11 †a | 0.15 | 0.037 | 0.046 |
| Females | | | | | |
| 0 | | 1.69 | 1.42 | 1.26 | 1.50 |
| | | 0.11 | 0.05 | 0.09 †a | 0.17 |
| 1 | | 1.21 | 1.32 | 1.13 | 1.01 |
| | | 0.13 | 0.12 | 0.09 †a | 0.07 †ab |
| 4 | | 1.33 | 1.55 | 0.933 | 1.12 |
| | | 0.06 | 0.07 | 0.053 †ab | 0.21 †a |
| 9 | | 1.46 *ab | 1.20 *a | 0.465 *b | 0.482 *b |
| | | 0.14 | 0.10 | 0.028 †b | 0.009 †ab |
| 15 | | 1.67 *ac | 1.40 *ac | 0.443 *bc | 0.411 *bc |
| | | 0.09 | 0.13 | 0.045 †b | 0.052 †b |

n = 36

The results of adrenic acid (22:4, n-6), the elongation product of AA, are shown in Table 8. Males of group 1 demonstrated an effect of time, increasing 33% from initial levels (Figure 2). Group differences occurred by week 15, whereas males of group 1 were different from groups 2, 3 and 4, in all of which 22:4 diminished as the study progressed. Like arachidonate, group differences only became significant at the last time interval.

The next fatty acid in the (n-6) series is 22:5. Figures 2, 4, 5, 6, 7, 8 and 9 indicate no time nor group significances for this fatty acid. However, Table 9 (Figure 3), illustrates that females fed diet 1, had elevated levels of this fatty acid at week 0. This group was fed the (n-6) precursors of this fatty acid; yet the erythrocyte content decreased with time. In fact, females of group 1 had inordinately elevated levels of 22:5 (n-6) at zero-time as compared to all other dietary groups. The erythrocytes of these animals and other dietary groups decreased in 22:5 with time. Group 1 and, to a degree group 2, were associated with more gradual declines compared to other groups.

Males fed diet 1 had previously shown group significant differences for shorter (n-6) fatty acids. None of these differences were translated into end products within 15 weeks. The results in Table 9 clearly indicate that 22:5 is a quantitatively minor erythrocyte component.

Table 10 provides a summation of (n-6) fatty acids in erythrocytes. As seen previously for 18:2, 20:4 and 22:4, males in group 1 showed a significant time effect by week 15. These effects culminated in a 31% increment in erythrocyte total (n-6) fatty acids for this group (Figure

Table 8: Results of Docosatetraenoic Acid (22:4, n-6) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscript are significantly different.

Significant orthogonal components are: 1, 2, and 4; linear and quadratic interaction with group.

Corresponding plots are provided in Figures 2 to 9.

Table 8:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.217 | 0.170 | 0.200 | 0.200 |
| | S.E.M. | 0.013 †a | 0.014 | 0.020 | 0.016 |
| 1 | | 0.128 | 0.114 | 0.104 | 0.112 |
| | | 0.013 †b | 0.024 | 0.011 | 0.014 |
| 4 | | 0.158 | 0.108 | 0.084 | 0.108 |
| | | 0.034 †a | 0.021 | 0.015 | 0.009 |
| 9 | | 0.167 | 0.074 | 0.032 | 0.037 |
| | | 0.025 †a | 0.010 | 0.006 | 0.010 |
| 15 | | 0.288 *a | 0.064 *b | 0.005 *b | 0.011 *b |
| | | 0.019 †a | 0.008 | 0.005 | 0.005 |
| Females | | | | | |
| 0 | | 0.247 | 0.206 | 0.165 | 0.243 |
| | | 0.028 | 0.016 | 0.013 | 0.023 |
| 1 | | 0.130 | 0.155 | 0.136 | 0.154 |
| | | 0.024 | 0.020 | 0.014 | 0.021 |
| 4 | | 0.163 | 0.152 | 0.080 | 0.152 |
| | | 0.014 | 0.020 | 0.007 | 0.037 |
| 9 | | 0.191 | 0.093 | 0.036 | 0.043 |
| | | 0.025 | 0.013 | 0.003 | 0.004 |
| 15 | | 0.261 *ab | 0.076 *b | 0.004 *b | 0.006 *b |
| | | 0.033 | 0.014 | 0.003 | 0.004 |

n = 36

Table 9: Results of Docosapentaenoic Fatty Acid (22:5, n-6) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscript are significantly different.

Significant orthogonal components are 1 and 2; linear and quadratic interaction with group.

Corresponding plots are provided in Figures 2 to 9.

Table 9:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.092 | 0.075 | 0.069 | 0.070 |
| | S.E.M. | 0.014 | 0.011 | 0.003 | 0.008 |
| 1 | | 0.043 | 0.038 | 0.049 | 0.048 |
| | | 0.010 | 0.006 | 0.012 | 0.012 |
| 4 | | 0.060 | 0.035 | 0.024 | 0.028 |
| | | 0.021 | 0.006 | 0.004 | 0.004 |
| 9 | | 0.040 | 0.033 | 0.012 | 0.011 |
| | | 0.006 | 0.002 | 0.001 | 0.002 |
| 15 | | 0.057 | 0.029 | 0.006 | 0.016 |
| | | 0.005 | 0.001 | 0.006 | 0.005 |
| Females | | | | | |
| 0 | | 0.118 | 0.079 | 0.078 | 0.094 |
| | | 0.014 †[a] | 0.005 | 0.013 | 0.019 |
| 1 | | 0.046 | 0.069 | 0.047 | 0.046 |
| | | 0.008 †[b] | 0.011 | 0.011 | 0.002 |
| 4 | | 0.040 | 0.042 | 0.026 | 0.045 |
| | | 0.004 †[b] | 0.005 | 0.002 | 0.006 |
| 9 | | 0.058 | 0.033 | 0.017 | 0.017 |
| | | 0.006 †[b] | 0.004 | 0.003 | 0.000 |
| 15 | | 0.061 | 0.029 | 0.010 | 0.010 |
| | | 0.011 †[b] | 0.009 | 0.004 | 0.006 |

n = 36

Table 10: Results of Summation of (n-6) Fatty Acids (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal components are 1, 2 and 4; linear and quadratic interaction with group; quadratic interaction with sex; and time interaction with sex (p=0.0447).

Corresponding plots are provided in Figures 2 to 9.

Table 10:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 3.46 | 3.85 | 3.55 | 3.18 |
| | S.E.M. | 0.19 †b | 0.39 | 0.32 | 0.34 †a |
| 1 | | 2.69 *ab | 3.21 *a | 1.96 *b | 2.16 *ab |
| | | 0.17 †b | 0.50 | 0.11 | 0.14 †ab |
| 4 | | 3.08 *a | 3.15 *a | 1.88 *b | 2.01 *b |
| | | 0.27 †b | 0.16 | 0.19 | 0.07 †b |
| 9 | | 2.77 *a | 3.30 *a | 1.24 *b | 1.17 *b |
| | | 0.08 †b | 0.15 | 0.04 | 0.08 †b |
| 15 | | 4.54 *a | 3.32 *b | 1.26 *c | 1.33 *c |
| | | 0.17 †a | 0.28 | 0.09 | 0.11 †b |
| Females | | | | | |
| 0 | | 4.01 | 3.37 | 2.94 | 3.50 |
| | | 0.21 | 0.15 | 0.16 †a | 0.39 |
| 1 | | 3.13 *a | 3.22 *a | 2.39 *ab | 2.33 *ab |
| | | 0.28 | 0.21 | 0.16 †ac | 0.18 †ac |
| 4 | | 3.27 *a | 3.61 *ab | 1.84 *b | 2.47 *ab |
| | | 0.08 | 0.09 | 0.08 †bc | 0.46 †a |
| 9 | | 3.45 *a | 2.87 *a | 1.21 *b | 1.31 *b |
| | | 0.30 | 0.24 | 0.07 †b | 0.05 †bc |
| 15 | | 3.94 *abc | 3.19 *b | 1.10 *c | 1.14 *c |
| | | 0.21 | 0.25 | 0.09 †b | 0.16 †b |

n = 36

2). The female counterparts, while not significantly different from the males in group 1, were also not different from the other dietary groups.

Groups 3 and 4, however, indicated a significant decrease in erythrocyte (n-6) fatty acids by the fourth week of study; by the 15th week, their (n-6) content was reduced to one-third of original levels (Figures 6 to 9). Again, the magnitude of the decreases in (n-6) fatty acids was twice that of any increases.

b) Effect on (n-3) Fatty Acids

Figures 10 to 17 portray the (n-3) family of fatty acid profiles for males and females; Tables 11 to 16 display each (n-3) fatty acid individually, for group comparison.

The results for the first (n-3) fatty acid, alpha-linolenic, are presented in Table 11. Significant differences were noted for group 2 only, which showed an increase from 0.0753 to 0.172 ug $18:3/10^6$ RBC in the males and an increase from 0.0572 to 0.154 ug $18:3/10^6$ RBC in the females in the first week of study. This represented increases of 2.7- and 2.3-fold in erythrocytes. There were no further significant increases with time after the first week; however the final 18:3 content corresponded to 182% and 208% increases from baseline levels, for males and females, respectively (Figures 12 and 13). Groups 3 and 4 were provided with low amounts of LNA, which showed a trend towards its depletion.

Table 12 displays the results for the next (n-3) fatty acid, 20:4, for which no dietary differences were detected. Although group 2 had previously shown a significant increase in 18:3, this metabolite did not accumulate to an appreciable extent; nor was it a major cellular

## Erythrocyte Fatty Acid Composition

Tables 11 to 16 represent individual (n-3) fatty acyl components of Cynomolgus monkey erythrocytes obtained by gas chromatographic analysis. Diet 1 contained lard and corn oil (high linoleic acid), Diet 2 contained lard and linseed oil (high linolenic acid) and Diets 3 and 4 contained lard, corn oil and menhaden oil (high long chain n-3). All diets were isocaloric and provided equal total polyunsaturate contents; however, Diet 1 provided (n-6), Diet 2 equal (n-6) to (n-3), Diets 3 and 4 equal (n-6) to longer chain (n-3) fatty acids, as the source of polyunsaturates. Diet 4 differed from Diet 3 by four-fold enrichment of alpha-tocopherol.

Table 11: Results of alpha-Linolenic Acid (18:3, n-3) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at week 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal component is linear; linear and quadratic interactions with group.

Corresponding plots are provided in Figures 10 to 17.

Table 11:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.023 | 0.075 | 0.030 | 0.017 |
| | S.E.M. | 0.011 | 0.018 †b | 0.011 | 0.003 |
| 1 | | 0.000 *b | 0.172 *a | 0.002 *b | 0.000 *b |
| | | 0.000 | 0.018 †a | 0.001 | 0.000 |
| 4 | | 0.003 *b | 0.191 *a | 0.001 *b | 0.004 *b |
| | | 0.003 | 0.010 †a | 0.002 | 0.003 |
| 9 | | 0.001 *b | 0.237 *a | 0.008 *b | 0.008 *b |
| | | 0.001 | 0.022 †a | 0.008 | 0.008 |
| 15 | | 0.000 *b | 0.212 *a | 0.000 *b | 0.049 *b |
| | | 0.000 | 0.024 †a | 0.000 | 0.031 |
| Females | | | | | |
| 0 | | 0.017 | 0.057 | 0.010 | 0.043 |
| | | 0.002 | 0.006 †b | 0.003 | 0.021 |
| 1 | | 0.000 *b | 0.154 *a | 0.000 *b | 0.000 *b |
| | | 0.000 | 0.011 †a | 0.000 | 0.000 |
| 4 | | 0.000 *b | 0.215 *a | 0.000 *b | 0.000 *b |
| | | 0.000 | 0.027 †a | 0.000 | 0.000 |
| 9 | | 0.000 *b | 0.205 *a | 0.000 *b | 0.007 *b |
| | | 0.000 | 0.030 †a | 0.000 | 0.006 |
| 15 | | 0.000 *b | 0.176 *a | 0.000 *b | 0.001 *b |
| | | 0.000 | 0.019 †a | 0.000 | 0.001 |

n = 36

Table 12: Results of Eicosatetraenoic Acid (20:4, n-3) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal component is linear; linear interaction with group; and sex-related significance at p=0.0469.

Corresponding plots are provided in Figures 10 to 17.

Table 12:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.011 | 0.013 | 0.014 | 0.016 |
| | S.E.M. | 0.001 | 0.003 | 0.002 | 0.002 |
| 1 | | 0.007 | 0.008 | 0.013 | 0.021 |
| | | 0.002 | 0.002 | 0.003 | 0.010 |
| 4 | | 0.004 | 0.031 | 0.021 | 0.022 |
| | | 0.001 | 0.008 | 0.003 | 0.017 |
| 9 | | 0.006 | 0.016 | 0.027 | 0.020 |
| | | 0.003 | 0.001 | 0.003 | 0.002 |
| 15 | | 0.000 | 0.032 | 0.026 | 0.033 |
| | | 0.000 | 0.003 | 0.001 | 0.002 |
| Females | | | | | |
| 0 | | 0.021 | 0.008 | 0.010 | 0.021 |
| | | 0.005 | 0.002 | 0.002 | 0.004 |
| 1 | | 0.002 | 0.023 | 0.014 | 0.026 |
| | | 0.001 | 0.012 | 0.001 | 0.015 |
| 4 | | 0.014 | 0.041 | 0.021 | 0.022 |
| | | 0.003 | 0.013 | 0.003 | 0.003 |
| 9 | | 0.017 | 0.040 | 0.027 | 0.025 |
| | | 0.011 | 0.010 | 0.005 | 0.003 |
| 15 | | 0.000 | 0.039 | 0.023 | 0.025 |
| | | 0.000 | 0.005 | 0.002 | 0.001 |

n = 36

component. Examination of the results at the 5% level of significance revealed that females of group 2 were different from males of groups 1, 2 and 4 and females of group 1, in the ninth week of study. The observed changes for males did not reach statistical significance.

Eicosapentaenoic acid (EPA, 20:5), the next metabolite in the (n-3) pathway, was significantly higher in groups 3 and 4 (Figures 14 to 17). Though none of these groups indicated an effect of diet within the first week, as was observed for LNA, by the fourth week, a marked response of erythrocyte 20:5 to the diet became apparent: males fed diet 3 increased 71.8%; females fed diet 3 increased 164%; males fed diet 4 increased 76.0% and females fed diet 4 increased 97.4%. The dietary fatty acids continued to have a drastic impact on erythrocyte fatty acid composition such that the final content of EPA reached 127%, 219%, 123% and 104% of baseline levels for males and females on diets 3 and 4, respectively (Table 13). Females in group 3, in particular, had a profound response to their diet; initial erythrocyte content of EPA was 0.434 and final content 1.39 ug/$10^6$ RBC, a 3.2-fold increase. The male counterparts, while also responsive to dietary manipulation (increasing 2.3-fold) required fifteen weeks to become significantly different from zero-time levels. Male and female monkeys fed diet 4 demonstrated a 2.2- and 2.0-fold accretion of EPA in erythrocytes.

Male monkeys of group 1 showed a decrease from 0.361 to 0.0433 ug EPA/$10^6$ RBC; females of group 1, levels decreased from 0.420 to 0.0296 ug EPA/$10^6$ RBC (Table 13). This represented a -88.0% and -93.0% change from zero-time, respectively.

Table 13: Results of Eicosapentaenoic Acid (20:5, n-3) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (•) significance at p=0.01. Means not sharing the same superscript are significantly different.

Significant orthogonal components are 1 and 4; group interaction with orthogonal components 1 and 4.

Corresponding plots are provided in Figures 10 to 17.

Table 13:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.361 | 0.445 | 0.632 | 0.695 |
| | S.E.M. | 0.017 | 0.074 | 0.053 †b | 0.040 †b |
| 1 | | 0.176 *bd | 0.268 *ad | 0.646 *ad | 0.674 *ac |
| | | 0.009 | 0.043 | 0.059 †b | 0.065 †b |
| 4 | | 0.116 *b | 0.257 *b | 1.09 *a | 1.22 *a |
| | | 0.012 | 0.026 | 0.12 †ab | 0.10 †a |
| 9 | | 0.050 *b | 0.269 *b | 1.14 *a | 1.11 *a |
| | | 0.013 | 0.019 | 0.02 †ab | 0.08 †ab |
| 15 | | 0.043 *b | 0.308 *b | 1.43 *a | 1.55 *a |
| | | 0.008 | 0.029 | 0.09 †a | 0.17 †a |
| Females | | | | | |
| 0 | | 0.420 | 0.279 | 0.434 | 0.699 |
| | | 0.070 | 0.059 | 0.432 †b | 0.101 †b |
| 1 | | 0.174 *bd | 0.200 *bcd | 0.724 *a | 0.734 *a |
| | | 0.022 | 0.038 | 0.036 †bc | 0.062 †bc |
| 4 | | 0.100 *b | 0.273 *b | 1.15 *a | 1.38 *a |
| | | 0.013 | 0.032 | 0.13 †ac | 0.28 †a |
| 9 | | 0.075 *b | 0.237 *b | 1.10 *a | 1.24 *a |
| | | 0.021 | 0.029 | 0.11 †ac | 0.07 †ac |
| 15 | | 0.030 *b | 0.275 *b | 1.39 *a | 1.42 *a |
| | | 0.007 | 0.007 | 0.12 †a | 0.16 †a |

n = 36

Group 2 was not associated with as extensive modifications of eicosapentaenoate levels; the males underwent a change of -30.8% and the females -1.4%. The divergent effects of the diets led to group differences as early as one week and terminated with both groups 3 and 4 being significantly different from both groups 1 and 2; groups 1 and 2 were not significantly different. Significant differences between groups occurred within one week, but within a group required up to 15 weeks. As was noted earlier, the effects of the diet were more obvious in females than in males.

Results for 22:5 (n-3) are presented in Table 14. No group nor time differences were detected at the 1% level of significance. At the 5% level of significance (results not indicated) females of group 2 were different at week 15 compared to weeks 0 and 1. This dietary group was also different from females in groups 1 and 3 at week 15. Examination of the results indicated all (n-3) groups experienced a slight to large increase in 22:5 (n-3): males group 2, +30%; females, +76%; males group 3, +0.94%; females, +23%; males group 4, +16% and females, +19%. The (n-3)-poor diet had net decreases: males group 1, -32%; females, -53%. Notably, diet 2 was associated with the largest increments of 22:5 (n-3), despite diets 3 and 4 providing small amounts of this fatty acid directly.

Docosahexaenoic acid (DHA, 22:6) increased significantly for groups 3 and 4 by week 4 (Figures 14 to 17). However, males of group 3 showed a significant change from week 1 concentrations at week 15 (Table 15). This group increased from 0.953 to 1.23 ug DHA/$10^6$ RBC, only a 29% difference from zero-time. Their female counterparts, demonstrated

Table 14: Results of Docosapentaenoic Acid (22:5, n-3) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal components are 1, 2, 3 and 4; group interaction with orthogonal components 1 and 4.

Corresponding plots are provided in Figures 10 to 17.

Table 14:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.292 | 0.302 | 0.320 | 0.302 |
| | S.E.M. | 0.017 | 0.033 | 0.019 | 0.020 |
| 1 | | 0.217 | 0.222 | 0.190 | 0.204 |
| | | 0.022 | 0.031 | 0.019 | 0.017 |
| 4 | | 0.245 | 0.304 | 0.286 | 0.316 |
| | | 0.030 | 0.032 | 0.048 | 0.013 |
| 9 | | 0.168 | 0.371 | 0.261 | 0.259 |
| | | 0.016 | 0.029 | 0.017 | 0.020 |
| 15 | | 0.199 | 0.393 | 0.323 | 0.349 |
| | | 0.024 | 0.018 | 0.011 | 0.041 |
| Females | | | | | |
| 0 | | 0.331 | 0.255 | 0.247 | 0.307 |
| | | 0.032 | 0.014 | 0.020 | 0.047 |
| 1 | | 0.215 | 0.220 | 0.220 | 0.220 |
| | | 0.020 | 0.018 | 0.009 | 0.019 |
| 4 | | 0.209 | 0.354 | 0.317 | 0.380 |
| | | 0.017 | 0.027 | 0.026 | 0.055 |
| 9 | | 0.184 | 0.355 | 0.257 | 0.307 |
| | | 0.040 | 0.026 | 0.023 | 0.020 |
| 15 | | 0.156 | 0.450 | 0.303 | 0.365 |
| | | 0.018 | 0.038 | 0.020 | 0.046 |

n = 36

Table 15: Results of Docosahexaenoic Acid (22:6, n-3) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal components are 1, 2, 3 and 4; group interactions with orthogonal components 1, 2 and 4; and time interaction with sex (p=0.043).

Corresponding plots are provided in Figures 10 to 17.

Table 15:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.681 | 0.733 | 0.953 | 0.751 |
| | S.E.M. | 0.033 | 0.086 | 0.128 †ab | 0.030 †bc |
| 1 | | 0.465 | 0.547 | 0.604 | 0.599 |
| | | 0.032 | 0.089 | 0.072 †b | 0.054 †b |
| 4 | | 0.509 *b | 0.490 *b | 1.01 *ab | 1.07 *a |
| | | 0.069 | 0.071 | 0.20 †ab | 0.04 †ac |
| 9 | | 0.359 *b | 0.480 *b | 0.990 *ab | 0.951 *a |
| | | 0.015 | 0.038 | 0.063 †ab | 0.068 †ab |
| 15 | | 0.483 *b | 0.420 *b | 1.23 *a | 1.27 *a |
| | | 0.024 | 0.041 | 0.10 †a | 0.14 †a |
| Females | | | | | |
| 0 | | 0.800 | 0.590 | 0.673 | 0.730 |
| | | 0.069 | 0.027 | 0.043 †b | 0.107 †b |
| 1 | | 0.537 | 0.487 | 0.739 | 0.665 |
| | | 0.047 | 0.033 | 0.038 †b | 0.053 †b |
| 4 | | 0.526 *b | 0.575 *b | 1.19 *a | 1.28 *a |
| | | 0.032 | 0.050 | 0.09 †a | 0.11 †a |
| 9 | | 0.443 *b | 0.414 *b | 1.01 *a | 1.10 *a |
| | | 0.053 | 0.026 | 0.09 †ab | 0.11 †ab |
| 15 | | 0.420 *b | 0.430 *b | 1.30 *a | 1.29 *a |
| | | 0.053 | 0.039 | 0.12 †a | 0.17 †a |

n = 36

significant difference at week 1 from weeks 4 and 15; DHA content was increased from 0.673 to 1.30 ug DHA/$10^6$ RBC, a change of 93%. Thus, females showed a 3-fold effect of diet compared to males in group 3.

At week 15, DHA for males in group 4, was significantly different from both week 0 and 1, while week 9 was not. This group also had an increase from 0.751 to 1.27 ug DHA/$10^6$ RBC, an increase of 69%. Similarly, females in group 4 indicated a significant change at weeks 4 and 15 versus weeks 0 and 1, with a lack of significance at week 9. Their DHA content rose from 0.730 to 1.29 ug/$10^6$ RBC during the study, a change of +77%.

Groups 1 and 2 showed no significant effect of time although a trend was seen with a gradual loss of initial DHA content. Even group 2, displayed decreases of 43% and 27% in males and females (Figures 12 and 13). Group 1 (Figures 10 and 11) showed decreases of 29% and 48%, which were similar to those seen in monkeys fed diet 2.

Differences among groups for DHA did not appear until week 4. At week 15, group 1 and 2 were not significantly different from each other but were different from groups 3 and 4. These significant changes were established and then maintained from week 4. Males of group 3 seemed to be an exception; they showed a diminished response to diet, especially when compared to their female counterparts. However, at the end of the study, their DHA content was 2.5- to 2.9-times greater than males fed diet 1 or diet 2. This group contrasted with females fed diet 3, which showed the greatest response to eicosapentaenoate as well as docosahexaenoate. Males of group 3 also demonstrated no significant change with time for total (n-6), as illustrated in Figure 6.

EPA and DHA appeared to follow similar time frames in erythrocytes: group differences for EPA were apparent in week 1 in females but were pronounced by week 4, while group differences for DHA appeared in week 4. For both EPA and DHA, 15 weeks were required for final concentrations to become significantly different from zero-time.

Table 16 provides a summation of the (n-3) family of fatty acid components. Groups 3 and 4 demonstrated significant effects of time. Males of group 3 showed an increase from week 1 total (n-3) content at week 15 but week 15 was not significantly different from week 0. For males in group 4, values increased significantly from week 1 to week 4 and increased thereafter. Both groups of males required 15 weeks to become different from zero-time levels of total (n-3). The female counterparts in groups 3 and 4 showed a more rapid incorporation of total (n-3) fatty acids, at week 4 (and 15), values were significantly different from week 0.

Although not statistically significant at the 1% level, results of group 1 showed a decrease of 47% and 62% in (n-3) fatty acids for males and females. For group 2, levels remained constant, with an average increase of 0.575%; males and females on diets 3 and 4 increased in total (n-3) content 54.4%, 119%, 82.2% and 72.2%, respectively. For groups 3 and 4, these increases were contrary to the two-thirds reduction observed for the total (n-6) fatty acids in these animals. Once again, females fed diet 3 showed a large margin over those fed diet 4 and twice the increase determined for their male counterparts. This observation may reflect the extraordinary incorporation of EPA seen previously for this group. Despite the smaller amplitude of their responses, males of group

Table 16: Results of Summation of (n-3) Fatty Acids (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal components are 1, 3 and 4; group interactions with orthogonal components 1 and 4; and quadratic interaction with sex (p=0.0307).

Corresponding plots are provided in Figures 10 to 17.

Table 16:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 1.37 | 1.57 | 1.95 | 1.78 |
| | S.E.M. | 0.05 | 0.19 | 0.20 †ab | 0.09 †bc |
| 1 | | 0.884 | 1.23 | 1.47 | 1.53 |
| | | 0.066 | 0.18 | 0.15 †b | 0.14 †b |
| 4 | | 0.882 *b | 1.29 *bc | 2.41 *ac | 2.63 *a |
| | | 0.105 | 0.12 | 0.37 †ab | 0.15 †ac |
| 9 | | 0.592 *b | 1.38 *bc | 2.44 *ab | 2.35 *ac |
| | | 0.041 | 0.07 | 0.08 †ab | 0.16 †ab |
| 15 | | 0.726 *b | 1.37 *b | 3.01 *a | 3.25 *a |
| | | 0.054 | 0.10 | 0.18 †a | 0.30 †a |
| Females | | | | | |
| 0 | | 1.59 | 1.20 | 1.37 | 1.81 |
| | | 0.16 | 0.09 | 0.09 †b | 0.28 †b |
| 1 | | 0.933 | 1.12 | 1.71 | 1.66 |
| | | 0.074 | 0.10 | 0.07 †bc | 0.12 †b |
| 4 | | 0.849 *b | 1.46 *bc | 2.67 *a | 3.06 *a |
| | | 0.051 | 0.08 | 0.22 †ac | 0.43 †a |
| 9 | | 0.744 *b | 1.26 *b | 2.40 *ac | 2.69 *a |
| | | 0.099 | 0.08 | 0.24 †ab | 0.20 †ab |
| 15 | | 0.605 *b | 1.37 *b | 3.01 *a | 3.11 *a |
| | | 0.077 | 0.07 | 0.26 †a | 0.37 †a |

n = 36

3 attained significant difference versus groups 1 and 2 by the end of the study.

Interestingly, erythrocytes of group 2, which doubled in LNA content, showed no net change in the derivatives of this fatty acid nor in the total (n-3) content. Group 1, fed an (n-3)-poor diet, also showed no significance difference from initial levels but ultimately contained one-half of the (n-3) fatty acids of group 2.

No significant differences among groups occurred until week 4, when a distinction between groups 3 and 4 versus group 1 occurred. These group differences were maintained through week 15 when groups 3 and 4 were different from both groups 1 and 2. By the end of the study, there was no significance, at the 1% level, for group 1 versus group 2. The final erythrocyte content of total (n-3) fatty acids for groups 3 and 4 were 4.6-times that of group 1 and 2.3-times that of group 2.

c) Effect on Ratio of Total (n-6) to Total (n-3) Fatty Acids

Table 17 (Figure 1) presents the ratio of total (n-6) fatty acids versus total (n-3) at each time interval during the study. Only males and females fed diet 1 demonstrated a significant difference with time when the results were expressed in this manner. Diet 1 also possessed a 64-fold enrichment of the total(n-6)/total(n-3) ratio, as compared to the other diets. For males fed diet 1, this ratio became significant by week 9 but indicated constant increases in (n-6) versus (n-3) fatty acids throughout the study. Females fed diet 1 had significantly higher values in the first week; with week 15 results significantly greater than all previous weeks. The ratio of total(n-6)/total(n-3) for males increased from 2.53 to 6.46 (155%); females increased from 2.59 to 6.80 (163%).

Table 17: Results of Summation of (n-6) / Summation of (n-3) Fatty Acids Analyses

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different.

Significant orthogonal components are quadratic and cubic; linear and quadratic interaction with group.

Corresponding plot is provided in Figure 1.

Table 17:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 2.53 | 2.47 | 1.83 | 1.79 |
| | S.E.M. | 0.14 †c | 0.07 | 0.11 | 0.08 |
| 1 | | 3.05 | 2.62 | 1.36 | 1.43 |
| | | 0.10 †c | 0.17 | 0.10 | 0.06 |
| 4 | | 3.58 *a | 2.48 *ab | 0.794 *b | 0.769 *b |
| | | 0.28 †bc | 0.17 | 0.041 | 0.045 |
| 9 | | 4.79 *a | 2.40 *b | 0.509 *b | 0.499 *b |
| | | 0.39 †b | 0.06 | 0.004 | 0.019 |
| 15 | | 6.46 *a | 2.43 *bc | 0.417 *bc | 0.413 *c |
| | | 0.69 †a | 0.05 | 0.016 | 0.012 |
| Females | | | | | |
| 0 | | 2.59 | 2.87 | 2.16 | 2.01 |
| | | 0.18 †c | 0.27 | 0.12 | 0.21 |
| 1 | | 3.37 | 2.96 | 1.39 | 1.41 |
| | | 0.24 †b | 0.32 | 0.07 | 0.10 |
| 4 | | 3.90 *a | 2.51 *ab | 0.703 *b | 0.798 *b |
| | | 0.21 †bc | 0.17 | 0.046 | 0.065 |
| 9 | | 4.84 *a | 2.27 *b | 0.513 *b | 0.490 *b |
| | | 0.53 †b | 0.07 | 0.024 | 0.021 |
| 15 | | 6.80 *a | 2.31 *b | 0.367 *c | 0.364 *c |
| | | 0.63 †a | 0.06 | 0.008 | 0.014 |

n = 36

Figure 1: Plot of Ratio of (n-6)/ (n-3) Fatty Acids vs. Time

Values represent comparison of group means +/- S.E.M. for total (n-6) vs total (n-3) fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Table 17.

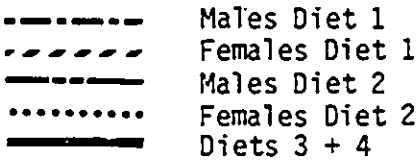
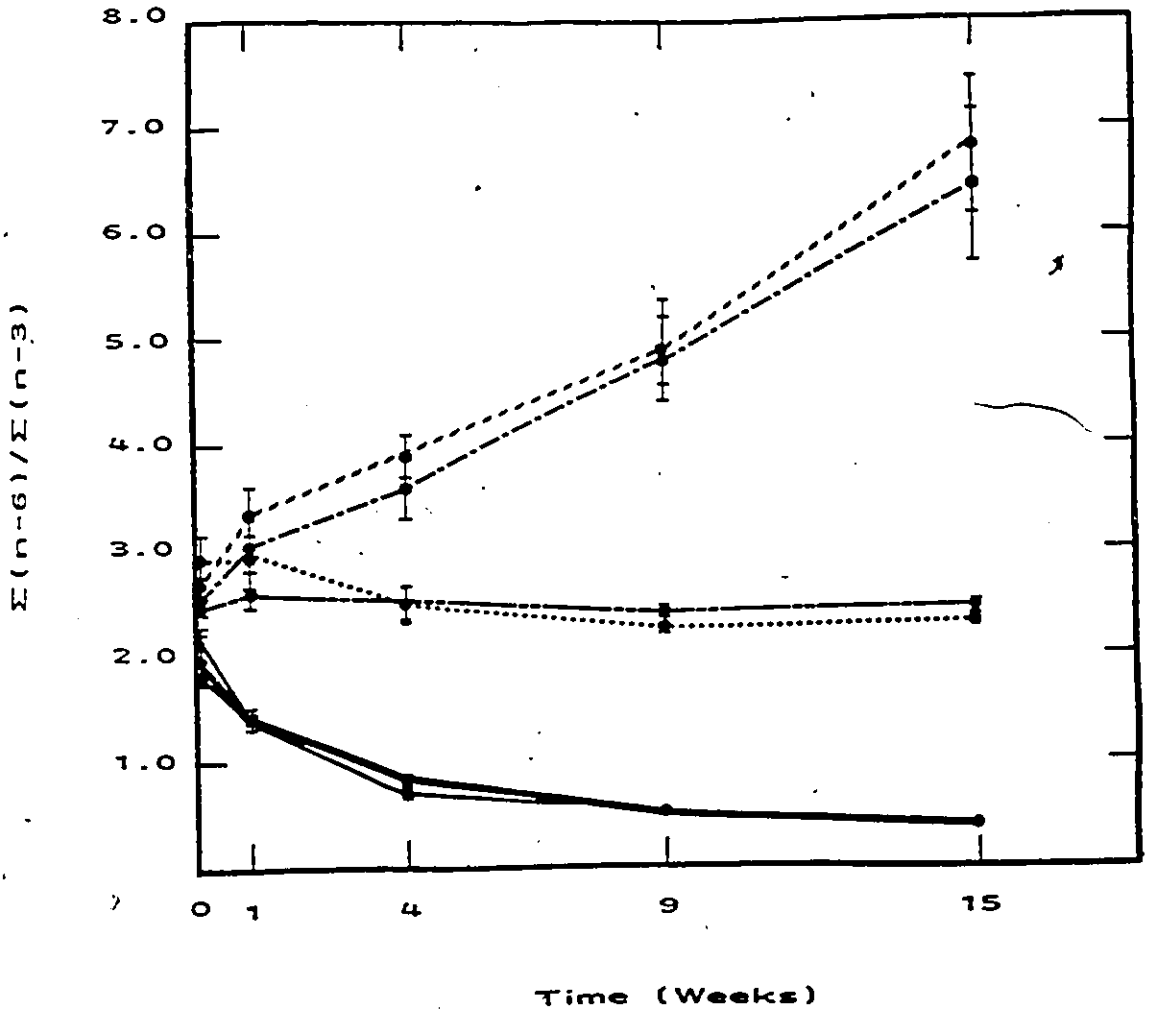


Figure 1



Animals in dietary group 2 were associated with smaller changes of -1.9% in the males and -19.4% in the females. The slight change in the ratio for group 2, which provided approximately equal amounts of (n-6) and (n-3) fatty acids, was a result of the previously witnessed diminution of longer chain (n-6) and lack of change in the (n-3) fatty acids, despite an elevation of LNA.

The results from groups 3 and 4 demonstrated greater changes in this ratio than from group 1; males and females in group 3 changed -77.2% and -83.0%, while males and females in group 4 changed -77.0% and -81.9%. Like diet 2, these diets provided approximately equal amounts of (n-6) and (n-3) fatty acids. For groups 3 and 4, the decline in the ratio of total(n-6)/total(n-3) was contributed by both a decrease in (n-6) of the numerator and by an increase in (n-3) of the denominator.

This ratio reached group significance for group 1 versus groups 3 and 4 by the fourth week of study; by the ninth week, group 1 was greater than groups 2, 3 and 4. In the 15th week, group 1 was still significantly greater than groups 2, 3 and 4; females of group 2 were significantly greater than males of group 4 and females of groups 3 and 4.

Although the females appeared to show a greater response to diets containing (n-3) fatty acids than males, these sex-related differences never reached significance.

Table 18 is a summary table of the results of the (n-6) and (n-3) fatty acids and their ratio. Percent changes in fatty acyl composition, for week 15 versus week 0, are presented for values for which time was significant.

Table 18: Summary Table of Results for (n-6) and (n-3) Fatty Acid Composition in Cynomolgus Erythrocytes

Values represent difference of week 15 results versus initial levels for each (n-6) and (n-3) fatty acid, for males and females. Only results for those fatty acids that indicated a significant time effect, p=0.01, are presented.

N.S.= no significant effect of time (↑) at p=0.01.

Table 18:

| Fatty Acid | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| 18:2 (n-6) | males | + 33 % | N.S. | - 53 % | - 46 % |
| | females | N.S. | N.S. | - 54 % | - 56 % |
| 20:4 (n-6) | | + 38 % | N.S. | N.S. | N.S. |
| | | N.S. | N.S. | - 65 % | - 73 % |
| 22:4 (n-6) | | + 33 % | N.S. | N.S. | N.S. |
| | | N.S. | N.S. | N.S. | N.S. |
| 22:5 (n-6) | | N.S. | N.S. | N.S. | N.S. |
| | | - 48 % | N.S. | N.S. | N.S. |
| $\Sigma$(n-6) | | + 31 % | N.S. | N.S. | - 58 % |
| | | N.S. | N.S. | - 63 % | - 67 % |
| 18:3 (n-3) | | N.S. | + 182 % | N.S. | N.S. |
| | | N.S. | + 208 % | N.S. | N.S. |
| 20:4 (n-3) | | N.S. | N.S. | N.S. | N.S. |
| | | N.S. | N.S. | N.S. | N.S. |
| 20:5 (n-3) | | N.S. | N.S. | + 127 % | + 123 % |
| | | N.S. | N.S. | + 219 % | + 104 % |
| 22:5 (n-3) | | N.S. | N.S. | N.S. | N.S. |
| | | N.S. | N.S. | N.S. | N.S. |
| 22:6 (n-3) | | N.S. | N.S. | + 29 % | + 69 % |
| | | N.S. | N.S. | + 93 % | + 77 % |
| $\Sigma$(n-3) | | N.S. | N.S. | + 54 % | + 82 % |
| | | N.S. | N.S. | + 119 % | + 72 % |
| $\Sigma$(n-6)/$\Sigma$(n-3) | | + 155 % | N.S. | N.S. | N.S. |
| | | + 163 % | N.S. | N.S. | N.S. |

d) Effect on Non-polyunsaturated Fatty Acids

Oleic acid (18:1, n-9) and total monounsaturates showed no significant group differences (Tables 19 and 20). Each diet provided approximately equal amounts of monounsaturates. Diets 1 and 2 provided monounsaturates predominantly as oleic acid; diets 3 and 4 provided oleic, palmitoleic (16:1) as well as gondoic (20:1, n-11) and cetoleic (22:1, n-11) acids, indigenous to fish oils (Table 5).

Tables 21 to 24 present the results for the saturated fatty acids palmitate (16:0), stearate (18:0) and arachidate (20:0) and the summation of saturated fatty acids: lauric (12:0), myristic (14:0), 16:0, heptadecanoic (17:0), 18:0, 20:0 and behenic (22:0). The results for palmitic acid (Table 21) and stearic acid (Table 22) indicated no group differences at the 1% level of significance. For all dietary groups a time effect was present, where weeks 1 and 9 were less than other time intervals. Red cell contents of arachidic acid (Table 23) were rather low. Only time made a significant difference; weeks 0 and 15 were less than other weeks and week 4 was elevated. When the summation of the saturates was examined (Table 24), no overall group differences existed. In this instance, time showed an effect; weeks 1 and 9 (similar to 16:0 and 18:0 results) were less than other weeks. With respect to diets, the only saturate to vary was stearic. Diets 3 and 4 provided half the amount compared to diets 1 and 2, although total saturates were marginally higher for the fish oil diets.

Thus, for the total monounsaturates and total saturates, no group differences were detected.

Table 19: Results of Oleic Acid (18:1, n-9) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing same superscripts are significantly different.

Significant orthogonal component is linear; linear and quadratic interaction with group; and quadratic interaction with sex.

Table 19:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 1.07 | 1.25 | 1.17 | 1.09 |
| | S.E.M. | 0.08 | 0.16 | 0.18 | 0.07 |
| 1 | | 0.999 | 1.15 | 0.777 | 0.980 |
| | | 0.111 | 0.13 | 0.062 | 0.103 |
| 4 | | 0.884 | 1.23 | 0.913 | 1.01 |
| | | 0.057 | 0.09 | 0.063 | 0.05 |
| 9 | | 0.893 | 1.38 | 0.767 | 0.801 |
| | | 0.041 | 0.183 | 0.057 | 0.049 |
| 15 | | 1.12 | 1.19 | 0.732 | 0.785 |
| | | 0.07 | 0.12 | 0.038 | 0.101 |
| Females | | | | | |
| 0 | | 1.34 | 0.994 | 0.946 | 1.10 |
| | | 0.10 | 0.031 | 0.049 | 0.14 |
| 1 | | 1.07 | 1.29 | 0.993 | 0.968 |
| | | 0.09 | 0.13 | 0.078 | 0.077 |
| 4 | | 1.01 | 1.31 | 0.931 | 1.19 |
| | | 0.07 | 0.06 | 0.015 | 0.21 |
| 9 | | 1.10 | 1.26 | 0.822 | 0.980 |
| | | 0.15 | 0.17 | 0.067 | 0.069 |
| 15 | | 0.955 | 1.06 | 0.699 | 0.724 |
| | | 0.041 | 0.12 | 0.062 | 0.110 |

n = 36

Table 20: Results of Summation of Monounsaturated Fatty Acid Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscript are significantly different.

Significant orthogonal components are 2 and 4; linear and quadratic interaction with group; and quadratic interaction with sex.

Table 20:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 1.38 | 1.57 | 1.52 | 1.41 |
| | S.E.M. | 0.05 | 0.18 | 0.13 | 0.06 |
| 1 | | 1.10 | 1.32 | 0.899 | 1.10 |
| | | 0.10 | 0.08 | 0.070 | 0.10 |
| 4 | | 1.01 | 1.47 | 1.04 | 1.12 |
| | | 0.07 | 0.18 | 0.07 | 0.05 |
| 9 | | 1.02 | 1.55 | 0.874 | 0.941 |
| | | 0.03 | 0.21 | 0.054 | 0.073 |
| 15 | | 1.81 | 1.77 | 1.16 | 1.35 |
| | | 0.20 | 0.23 | 0.07 | 0.17 |
| Females | | | | | |
| 0 | | 1.72 | 1.29 | 1.20 | 1.50 |
| | | 0.11 | 0.04 | 0.05 | 0.16 |
| 1 | | 1.24 | 1.46 | 1.14 | 1.16 |
| | | 0.09 | 0.13 | 0.08 | 0.10 |
| 4 | | 1.22 | 1.51 | 1.09 | 1.40 |
| | | 0.08 | 0.06 | 0.03 | 0.25 |
| 9 | | 1.37 | 1.41 | 0.976 | 1.10 |
| | | 0.19 | 0.18 | 0.044 | 0.07 |
| 15 | | 1.49 | 1.49 | 1.22 | 1.26 |
| | | 0.07 | 0.08 | 0.18 | 0.19 |

n = 36

Table 21: Results of Palmitic Acid (16:0) Analyses ($ug/10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different; (†) pertains to males and females of all diets combined.

Significant orthogonal components are 1 and 4; quadratic interaction with sex. No group nor group by time interaction.

Table 21:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 †a | mean | 2.00 | 2.38 | 2.42 | 2.09 |
| | S.E.M. | 0.07 | 0.29 | 0.33 | 0.11 |
| 1 †b | | 1.80 | 2.01 | 1.58 | 1.75 |
| | | 0.18 | 0.30 | 0.13 | 0.09 |
| 4 †a | | 1.94 | 2.17 | 2.03 | 2.28 |
| | | 0.13 | 0.16 | 0.09 | 0.10 |
| 9 †b | | 1.62 | 2.11 | 1.93 | 1.81 |
| | | 0.06 | 0.15 | 0.16 | 0.18 |
| 15 †a | | 2.34 | 2.06 | 2.29 | 2.37 |
| | | 0.15 | 0.16 | 0.13 | 0.33 |
| Females | | | | | |
| 0 | | 2.36 | 1.97 | 1.96 | 2.33 |
| | | 0.20 | 0.22 | 0.07 | 0.23 |
| 1 | | 2.01 | 2.08 | 2.00 | 1.99 |
| | | 0.11 | 0.14 | 0.11 | 0.12 |
| 4 | | 1.94 | 2.17 | 2.25 | 2.73 |
| | | 0.07 | 0.13 | 0.15 | 0.47 |
| 9 | | 1.86 | 1.90 | 2.11 | 2.22 |
| | | 0.18 | 0.18 | 0.12 | 0.15 |
| 15 | | 2.08 | 2.00 | 2.12 | 2.28 |
| | | 0.15 | 0.21 | 0.10 | 0.42 |

n = 36

Table 22: Results of Stearic Acid (18:0) Analyses ($ug/10^6$ RBC)

Values represent group means +/-S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing the same superscripts are significantly different; (†) pertains to males and females of all diets combined.

Significant orthogonal components are 2 and 4; quadratic interaction with sex (p=0.024) and group interaction with sex (p=0.0542). No group nor group by time interactions.

Table 22:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 †a | mean | 1.37 | 1.60 | 1.35 | 1.37 |
| | S.E.M. | 0.09 | 0.21 | 0.16 | 0.09 |
| 1 †b | | 1.10 | 1.43 | 0.908 | 1.06 |
| | | 0.13 | 0.22 | 0.114 | 0.04 |
| 4 †a | | 1.32 | 1.55 | 1.20 | 1.33 |
| | | 0.12 | 0.14 | 0.17 | 0.03 |
| 9 †b | | 0.963 | 1.50 | 1.07 | 1.02 |
| | | 0.047 | 0.05 | 0.07 | 0.07 |
| 15 †a | | 1.63 | 1.55 | 1.29 | 1.41 |
| | | 0.07 | 0.13 | 0.11 | 0.15 |
| Females | | | | | |
| 0 | | 1.48 | 1.28 | 1.24 | 1.42 |
| | | 0.07 | 0.13 | 0.06 | 0.16 |
| 1 | | 1.29 | 1.12 | 1.15 | 1.19 |
| | | 0.15 | 0.05 | 0.10 | 0.07 |
| 4 | | 1.31 | 1.65 | 1.27 | 1.66 |
| | | 0.05 | 0.06 | 0.07 | 0.33 |
| 9 | | 1.20 | 1.24 | 1.02 | 1.17 |
| | | 0.11 | 0.06 | 0.07 | 0.06 |
| 15 | | 1.44 | 1.42 | 1.23 | 1.25 |
| | | 0.06 | 0.07 | 0.10 | 0.15 |

n = 36

Table 23: Results of Arachidic Acid (20:0) Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01. Means not sharing same superscripts are significantly different; (†) pertains to males and females of all diets combined.

Significant orthogonal components are 2 and 4; no group nor group by time interaction.

Table 23:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 †c | mean | 0.056 | 0.047 | 0.023 | 0.024 |
| | S.E.M. | 0.037 | 0.021 | 0.003 | 0.001 |
| 1 †b | | 0.049 | 0.063 | 0.051 | 0.055 |
| | | 0.054 | 0.009 | 0.009 | 0.004 |
| 4 †a | | 0.073 | 0.066 | 0.073 | 0.075 |
| | | 0.010 | 0.018 | 0.010 | 0.009 |
| 9 †b | | 0.044 | 0.071 | 0.060 | 0.042 |
| | | 0.003 | 0.003 | 0.007 | 0.008 |
| 15 †c | | 0.027 | 0.025 | 0.029 | 0.048 |
| | | 0.002 | 0.002 | 0.001 | 0.012 |
| Females | | | | | |
| 0 | | 0.038 | 0.023 | 0.033 | 0.049 |
| | | 0.006 | 0.005 | 0.006 | 0.011 |
| 1 | | 0.061 | 0.061 | 0.062 | 0.047 |
| | | 0.005 | 0.004 | 0.002 | 0.005 |
| 4 | | 0.075 | 0.091 | 0.070 | 0.095 |
| | | 0.007 | 0.007 | 0.007 | 0.016 |
| 9 | | 0.068 | 0.062 | 0.042 | 0.058 |
| | | 0.010 | 0.009 | 0.007 | 0.004 |
| 15 | | 0.023 | 0.024 | 0.027 | 0.058 |
| | | 0.003 | 0.002 | 0.003 | 0.035 |

n = 36

Table 24: Results of Summation of Saturated Fatty Acid Analyses (ug/$10^6$ RBC)

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (•) significance at p=0.01. Means not sharing the same superscripts are significantly different; (†) pertains to males and females of all diets combined.

Significant orthogonal components are 2 and 4; quadratic interaction with sex. No group nor group by time interactions.

Table 24:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 †a | mean | 3.51 | 4.07 | 3.87 | 3.55 |
| | S.E.M. | 0.11 | 0.53 | 0.51 | 0.17 |
| 1 †b | | 3.03 | 3.57 | 2.60 | 2.94 |
| | | 0.29 | 0.53 | 0.17 | 0.07 |
| 4 †a | | 3.39 | 3.89 | 3.38 | 3.77 |
| | | 0.25 | 0.28 | 0.25 | 0.13 |
| 9 †b | | 2.68 | 3.75 | 3.11 | 2.94 |
| | | 0.05 | 0.19 | 0.12 | 0.25 |
| 15 †a | | 4.09 | 3.73 | 3.72 | 3.92 |
| | | 0.23 | 0.32 | 0.22 | 0.47 |
| Females | | | | | |
| 0 | | 3.97 | 3.22 | 3.29 | 3.87 |
| | | 0.28 | 0.25 | 0.10 | 0.36 |
| 1 | | 3.44 | 3.17 | 3.28 | 3.30 |
| | | 0.27 | 0.13 | 0.18 | 0.17 |
| 4 | | 3.39 | 3.98 | 3.68 | 4.56 |
| | | 0.06 | 0.14 | 0.23 | 0.82 |
| 9 | | 3.21 | 3.27 | 3.21 | 3.52 |
| | | 0.28 | 0.21 | 0.20 | 0.21 |
| 15 | | 3.61 | 3.55 | 3.49 | 3.79 |
| | | 0.18 | 0.29 | 0.21 | 0.56 |

n = 36

## ii: Effect of Dietary (n-3) on Other Haematological Parameters

a) Superoxide Dismutase Activity in Erythrocytes

The superoxide dismutase (SOD) results (Table 25) indicated a lack of overall group or sex-related significances, at the 5% level. Time, however, was significant at p=0.01. Week 9 SOD values were elevated compared to all other weeks; week 15 values were significantly less than weeks 0, 4 and 9. With time, some group interactions occurred; yet the respective replicate sizes were prohibitively small for further statistical analysis. Females fed diet 3 appeared to have elevated SOD activity in the 15th week.

b) Glutathione Peroxidase Activity in Erythrocytes

The glutathione peroxidase (GSH-Px) results (Table 26) also did not elucidate any group effects. Group 2 demonstrated a significant effect of sex, where females were 1.75-times greater than their male counterparts, at week 0. Time was significant; week 4 was greater than all other weeks (p=0.01).

c) Thiobarbituric Acid Assay of Whole Blood

The thiobarbituric acid (TBA) assay (Table 27), to detect the presence of peroxidized products in the red cell, revealed a significant time by group interaction. The highest values occurred in group 2 at week 0. At week 4, females of groups 2 and 4 levels were significantly higher than group 1 by up to 49.0% and to themselves at weeks 1, 9 and 15.

d) Platelet Cell Counts

Platelet cell counts, which affect the haemostatic response, showed a group effect (p=0.0645) and a group by time interaction

Table 25: SOD Activity in Erythrocytes
(units/mg haemoglobin x $10^{-1}$)

Superoxide dismutase activity present in supernatant of lysed extracted erythrocytes from weeks 0, 4, 9 and 15. Means +/- S.E.M. derived from standard curve of purified SOD, determined on the Abbott Discrete Analyzer.

No significant group effects, $p<0.05$. Time significant (†), $p=0.000$ and time by group significant, $p=0.000$. Time intervals not sharing same superscripts are significantly different; superscripts pertain to males and females of all diet combined.

Significant orthogonal components are 2, 3; quadratic interaction with group and cubic interaction with group.

Table 25:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 †b | mean | 230 | 212 | 241 | 250 |
| | S.E.M. | 19 | 17 | 14 | 32 |
| 4 †b | | 184 | 307 | 171 | 248 |
| | | 19 | 29 | 4 | 27 |
| 9 †a | | 369 | 383 | 298 | 306 |
| | | 51 | 22 | 29 | 31 |
| 15 †c | | 191 | 180 | 184 | 207 |
| | | 21 | 8 | 8 | 29 |
| Females | | | | | |
| 0 | | 207 | 175 | 255 | 225 |
| | | 30 | 21 | 27 | 20 |
| 4 | | 186 | 220 | 223 | 204 |
| | | 19 | 36 | 32 | 32 |
| 9 | | 306 | 342 | 363 | 328 |
| | | 38 | 37 | 30 | 33 |
| 15 | | 186 | 167 | 250 | 196 |
| | | 26 | 25 | 21 | 15 |

n = 37

Table 26: GSH-Px Activity in Erythrocytes
(units/mg haemoglobin x 10)

Glutathione peroxidase (GSH-Px) activity present in erythrocyte lysates with haemoglobin stabilized to cyanmethemoglobin form. Indirect assay involves decrease in absorbance, at 340 nm, as NADPH is converted to $NADP^+$. Assay completed using Abbott Discrete Analyzer.

Means +/- S.E.M. for four dietary groups, for weeks 0, 4, 9 and 15 of study.

No group significance; sex significant (*), p=0.0259; time (†) significant, p=0.000 and sex by time significant, p=0.000. '†' pertains to males and females of all diets combined.

Significant orthogonal components are 1, 2, 3; linear interaction with sex and quadratic interaction with sex.

Table 26:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| **Males** | | | | | |
| 0 †b | mean | 213 | 203 •b | 207 | 226 |
| | S.E.M. | 23 | 14 | 22 | 14 |
| 4 †a | | 319 | 272 | 302 | 317 |
| | | 14 | 16 | 11 | 27 |
| 9 †b | | 261 | 248 | 254 | 267 |
| | | 6 | 14 | 29 | 14 |
| 15 †b | | 271 | 277 | 253 | 243 |
| | | 7 | 16 | 23 | 27 |
| **Females** | | | | | |
| 0 | | 320 | 353 •a | 308 | 305 |
| | | 51 | 25 | 20 | 37 |
| 4 | | 360 | 285 | 323 | 333 |
| | | 45 | 14 | 23 | 65 |
| 9 | | 320 | 251 | 268 | 249 |
| | | 45 | 11 | 11 | 9 |
| 15 | | 309 | 283 | 208 | 225 |
| | | 57 | 34 | 10 | 21 |

n = 37

Table 27: Thiobarbituric Acid (TBA) Assay on Whole Blood (mol x $10^{-9}$ MDA/ $10^{6}$ RBC)

Means +/- S.E.M. derived from standard curve for four dietary groups at weeks 0, 1, 4, 9 and 15 of study. Values represent triplicate determinations for each animal, for a chromagen formed when thiobarbituric acid complexes with malondialdehyde (MDA) at 532 nm.

Statistical significances include group (*), p=0.0002, time (†), p=0.0000, group by time, p=0.0000 and time by sex, p=0.0128. Means not sharing same superscripts are significantly different.

Significant orthogonal components are 1, 2, 3, 4; 1-, 2-, 3-, 4-interactions with group and 2-, 3-interactions with sex.

Table 27:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 0.405 *b | 0.518 *a | 0.362 *b | 0.333 *b |
| | S.E.M. | 0.019 | 0.037 †a | 0.027 | 0.020 |
| 1 | | 0.269 | 0.316 | 0.291 | 0.348 |
| | | 0.026 | 0.012 †b | 0.018 | 0.018 |
| 4 | | 0.301 *b | 0.362 *ab | 0.381 *ab | 0.391 *ab |
| | | 0.014 | 0.034 †b | 0.029 | 0.013 |
| 9 | | 0.239 | 0.256 | 0.288 | 0.284 |
| | | 0.023 | 0.019 †b | 0.011 | 0.017 |
| 15 | | 0.331 | 0.366 | 0.398 | 0.355 |
| | | 0.021 | 0.018 †b | 0.031 | 0.018 |
| Females | | | | | |
| 0 | | 0.357 *b | 0.519 *a | 0.216 *b | 0.350 *b |
| | | 0.018 | 0.028 †a | 0.045 | 0.041 †a |
| 1 | | 0.224 | 0.301 | 0.299 | 0.320 |
| | | 0.010 | 0.033 †b | 0.014 | 0.012 †b |
| 4 | | 0.311 *b | 0.447 *a | 0.386 *ab | 0.449 *a |
| | | 0.021 | 0.025 †a | 0.031 | 0.054 †a |
| 9 | | 0.231 | 0.252 | 0.252 | 0.272 |
| | | 0.014 | 0.013 †b | 0.017 | 0.020 †b |
| 15 | | 0.291 | 0.323 | 0.321 | 0.394 |
| | | 0.038 | 0.037 †b | 0.020 | 0.054 †a |

n = 40

Table 28: Platelet Cell Count
per 100 uL Whole Blood (x $10^3$)

Mean +/- S.E.M. cell counts of citrated whole blood as determined on a Coulter Counter (Model S Plus IV) at weeks 0, 1, 4, 6, 9, 12 and 15 of the study.

No significant group effect; effect of time was significant (†). Weeks not sharing same superscript are significantly different ($p>0.05$); superscripts pertain to males and females of all diets combined.

Significant orthogonal components are 1, 2, 3, 6; linear interaction with group and cubic interaction with group.

Table 28:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| **Males** | | | | | |
| 0 †a | mean | 581 | 519 | 516 | 453 |
| | S.E.M. | 46 | 62 | 75 | 38 |
| 1 †b | | 492 | 403 | 538 | 423 |
| | | 29 | 46 | 70 | 26 |
| 4 †b | | 455 | 394 | 535 | 398 |
| | | 25 | 56 | 69 | 17 |
| 6 †c | | 409 | 380 | 516 | 362 |
| | | 30 | 50 | 65 | 15 |
| 9 †b | | 450 | 415 | 532 | 432 |
| | | 37 | 55 | 46 | 23 |
| 12 †bc | | 454 | 379 | 516 | 429 |
| | | 33 | 41 | 63 | 30 |
| 15 †bc | | 415 | 438 | 492 | 395 |
| | | 20 | 45 | 37 | 44 |
| **Females** | | | | | |
| 0 | | 511 | 446 | 544 | 365 |
| | | 34 | 50 | 52 | 22 |
| 1 | | 463 | 397 | 495 | 368 |
| | | 51 | 43 | 56 | 36 |
| 4 | | 493 | 378 | 456 | 453 |
| | | 54 | 40 | 35 | 99 |
| 6 | | 447 | 362 | 434 | 338 |
| | | 54 | 40 | 37 | 29 |
| 9 | | 452 | 402 | 474 | 352 |
| | | 47 | 56 | 47 | 33 |
| 12 | | 413 | 385 | 454 | 385 |
| | | 50 | 42 | 43 | 45 |
| 15 | | 441 | 398 | 451 | 398 |
| | | 49 | 39 | 39 | 54 |

n = 39

(p=0.0596)(Table 28). This indicated that with the progress of the study, certain group(s) exhibited an effect on platelet counts. In most cases, platelet counts decreased with time. Week 0 platelet counts were the highest; the levels established at week 6 were maintained thereafter.

e) Bleeding Times

Bleeding times (Table 29) exhibited significant differences with time; the first three intervals (weeks 1, 4, 6) were different from the latter time points (weeks 9 and 12). That is, a decrease in bleeding time occurred by week 9, which was maintained to week 12. All groups showed a decrease in bleeding times as the study progressed. Inspection of the S.E.M.s indicated a large degree of variability among monkeys which reduces statistical significances.

f) Bleeding Time Blood Volume

The bleeding time blood volume (BTBV) results (Table 30) were similar to those of bleeding time. No group nor sex-related differences occurred although an effect of time was present. Initial values were significantly higher than most remaining weeks (excepting week 6), decreasing linearly with time. The results indicated that the largest volume of blood loss coincided with the longest bleeding times; both parameters showed no evidence of dietary manipulation.

g) Body Weights

Body weights of males (Table 31) and of females (Table 32) were used to ascertain adequate intakes of all diets. Analysis of variance with a repeated measures design demonstrated monkey weights were variable throughout the study. Since no statistical significance was identified for group, sex or time, one cannot establish any diet-related effect on

Table 29: Simplate Bleeding Times (min.)

Bleeding time, in minutes, represents the combined haemostatic response to the making of a uniform small incision and the time required for cessation of bleeding.

Means +/- S.E.M. for all dietary groups on weeks 1, 4, 6, 9 and 12 of study.

No group nor sex significances; time was significant (†), p=0.0002. Time intervals not sharing same superscripts are significantly different; superscripts pertain to males and females of all diets combined.

Significant orthogonal components indicated a linear trend, including an interaction with group by sex.

Table 29:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 1 †a | mean | 5.10 | 5.00 | .5.65 | 5.80 |
| | S.E.M. | 0.73 | 0.85 | 0.93 | 0.96 |
| 4 †a | | 3.90 | 5.05 | 4.75 | 5.35 |
| | | 0.52 | 1.16 | 0.94 | 1.45 |
| 6 †a | | 3.95 | 4.60 | 5.95 | 5.00 |
| | | 0.35 | 1.23 | 1.24 | 0.67 |
| 9 †b | | 4.15 | 3.20 | 4.10 | 5.05 |
| | | 0.47 | 0.38 | 0.17 | 1.09 |
| 12 †b | | 3.95 | 3.45 | 4.10 | 4.00 |
| | | 0.87 | 0.26 | 0.32 | 1.01 |
| Females | | | | | |
| 1 | | 7.10 | 4.55 | 3.70 | 3.90 |
| | | 1.54 | 0.46 | 0.50 | 0.64 |
| 4 | | 4.60 | 3.40 | 4.25 | 5.25 |
| | | 0.55 | 0.40 | 0.75 | 1.16 |
| 6 | | 4.65 | 4.05 | 3.70 | 5.60 |
| | | 0.84 | 0.99 | 0.18 | 0.89 |
| 9 | | 4.15 | 3.00 | 3.10 | 4.70 |
| | | 0.58 | 0.35 | 0.33 | 0.59 |
| 12 | | 3.70 | 3.45 | 3.20 | 4.45 |
| | | 0.68 | 0.57 | 0.30 | 0.44 |

n = 40

Table 30: Bleeding Time Blood Volume (uL)

Means +/- S.E.M. derived from eluates of whole blood in stabilized cyanmethaemoglobin form. Absorbance readings (418 nm), in triplicate, were converted to corresponding blood volumes, from the equation of the individual standard curves.

No group- nor sex- related significances; time effect was significant (†), p=0.0148. Times not sharing same superscripts are significantly different; superscripts pertain to males and females of all diets combined.

Significant orthogonal component was 1.

Table 30:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 1 †a | mean | 177 | 291 | 394 | 382 |
| | S.E.M. | 109 | 155 | 197 | 236 |
| 4 †b | | 65.7 | 182 | 208 | 356 |
| | | 9.6 | 89 | 109 | 195 |
| 6 †ab | | 72.6 | 147 | 351 | 227 |
| | | 12.1 | 67 | 211 | 109 |
| 9 †b | | 117 | 111 | 237 | 297 |
| | | 16 | 32 | 68 | 202 |
| 12 †b | | 130 | 126 | 164 | 196 |
| | | 30 | 31 | 49 | 128 |
| Females | | | | | |
| 1 | | 466 | 169 | 93.5 | 156 |
| | | 108 | 32 | 32.3 | 63 |
| 4 | | 151 | 81.6 | 99.6 | 269 |
| | | 31 | 27.4 | 52.8 | 164 |
| 6 | | 113 | 163 | 134 | 400 |
| | | 19 | 113 | 33 | 238 |
| 9 | | 115 | 102 | 64.4 | 274 |
| | | 36 | 37 | 23.6 | 126 |
| 12 | | 176 | 182 | 72.3 | 245 |
| | | 72 | 115 | 9.2 | 96 |

n = 40

Table 31: Body Weights (kg)

Means +/- S.E.M. body weights of males, weeks 0 to 13 of study.

No group statistical significance; sex was significant (p=0.000); time was significant (p=0.000); time interaction with sex (p=0.000) and time interaction with group (p=0.0198). However, upon application of post hoc test, no particular significances could be identified at the 5% level.

Significant orthogonal components: 1, 5, 6, 8, 9, 10; 1-, 3-, 10-interaction with sex; 3-, 5-, 9-interaction with group and 10-interaction with group by sex.

Table 31:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | | | | | |
| 0 | mean | 4.65 | 4.23 | 4.31 | 3.98 |
| | S.E.M. | 0.34 | 0.17 | 0.25 | 0.26 |
| 1 | | 4.63 | 4.20 | 4.40 | 4.03 |
| | | 0.37 | 0.19 | 0.22 | 0.29 |
| 3 | | 4.73 | 4.22 | 4.30 | 4.02 |
| | | 0.33 | 0.16 | 0.24 | 0.30 |
| 4 | | 4.59 | 4.20 | 4.30 | 3.99 |
| | | 0.32 | 0.20 | 0.24 | 0.28 |
| 5 | | 4.56 | 4.21 | 4.24 | 3.99 |
| | | 0.32 | 0.20 | 0.26 | 0.27 |
| 6 | | 4.78 | 4.30 | 4.60 | 4.24 |
| | | 0.31 | 0.22 | 0.29 | 0.33 |
| 7 | | 4.73 | 4.25 | 4.46 | 4.10 |
| | | 0.28 | 0.20 | 0.22 | 0.27 |
| 8 | | 4.70 | 4.26 | 4.44 | 4.02 |
| | | 0.28 | 0.21 | 0.27 | 0.25 |
| 9 | | 4.59 | 4.18 | 4.44 | 4.12 |
| | | 0.28 | 0.21 | 0.25 | 0.29 |
| 10 | | 4.80 | 4.34 | 4.64 | 4.26 |
| | | 0.29 | 0.23 | 0.27 | 0.30 |
| 11 | | 4.64 | 4.27 | 4.44 | 4.21 |
| | | 0.27 | 0.22 | 0.26 | 0.29 |
| 12 | | 4.76 | 4.30 | 4.47 | 4.22 |
| | | 0.29 | 0.20 | 0.26 | 0.28 |
| 13 | | 4.71 | 4.29 | 4.40 | 4.18 |
| | | 0.28 | 0.20 | 0.24 | 0.27 |

n = 20

Table 32: Body Weights (kg)

Means +/- S.E.M. body weights of females, weeks 0 to 13 of study.

Refer to Table 12 for statistical significances.

Table 32:

| Week | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Females | | | | | |
| 0 | mean | 3.24 | 3.18 | 3.23 | 3.15 |
| | S.E.M. | 0.19 | 0.18 | 0.11 | 0.17 |
| 1 | | 3.24 | 3.33 | 3.30 | 3.15 |
| | | 0.19 | 0.17 | 0.17 | 0.17 |
| 3 | | 3.38 | 3.36 | 3.22 | 3.14 |
| | | 0.25 | 0.17 | 0.15 | 0.18 |
| 4 | | 3.30 | 3.30 | 3.07 | 3.08 |
| | | 0.21 | 0.18 | 0.12 | 0.18 |
| 5 | | 3.27 | 3.26 | 3.18 | 3.08 |
| | | 0.21 | 0.17 | 0.10 | 0.15 |
| 6 | | 3.33 | 3.29 | 3.22 | 3.28 |
| | | 0.27 | 0.21 | 0.15 | 0.18 |
| 7 | | 3.35 | 3.30 | 3.20 | 3.21 |
| | | 0.24 | 0.22 | 0.11 | 0.16 |
| 8 | | 3.32 | 3.16 | 3.10 | 3.18 |
| | | 0.22 | 0.21 | 0.10 | 0.16 |
| 9 | | 3.22 | 3.11 | 3.11 | 3.12 |
| | | 0.22 | 0.21 | 0.10 | 0.15 |
| 10 | | 3.34 | 3.21 | 3.17 | 3.21 |
| | | 0.24 | 0.23 | 0.11 | 0.18 |
| 11 | | 3.39 | 3.19 | 3.08 | 3.12 |
| | | 0.32 | 0.22 | 0.14 | 0.15 |
| 12 | | 3.30 | 3.18 | 3.14 | 3.20 |
| | | 0.25 | 0.20 | 0.13 | 0.14 |
| 13 | | 3.37 | 3.29 | 3.23 | 3.26 |
| | | 0.26 | 0.21 | 0.11 | 0.14 |

n = 20

intakes. The somewhat elevated weight of males fed diet 1 throughout the study pertained to one heavier animal which had to be substituted prior to the study from a group of surplus males.

h) Serum Cholesterol and Triglyceride Levels

Tables 33 to 36 provide serum cholesterol (total and HDL), triglyceride and the ratio of triglyceride to total cholesterol results. Total cholesterol results (Table 33) depicted a sex-related significant difference in which females in group 2 possessed 34% higher total cholesterol compared to their males counterparts. In this instance, the significance appeared to be attributed to an elevation of total cholesterol in the females rather than abnormally low levels in the males. Several of the S.E.M.s were of appreciable magnitude, reflecting the heterogeneity of the monkeys in this response. The HDL fraction of cholesterol (Table 34) revealed that males of group 3 were significantly lower than either females of group 2 and males of group 1, by 48.0%. The difference between males and females of group 3 HDL was not significant from each other, nor were the females significantly different from other groups. Secondly, the similar diet, fed to group 4, did not demonstrate the differences determined for the males in group 3. This diminution of HDL did not correspond to a significantly lowered total cholesterol for males in group 3, in Table 33. The sex-related significance of group 2, Table 33, was not isolated in the HDL fraction, Table 34. When the serum triglyceride component was examined (Table 35), no significant group- nor sex-related differences were found. The results, expressed as a ratio of triglycerides to total cholesterol (Table 36), ranged from 0.256 (males in group 3) to a maximum of 0.400 (males in group 2). Neither total

## Serum Cholesterol and Triglyceride Levels

Tables 33 through 36 represent serum cholesterol and triglyceride levels of males and females, determined using the Abbott A-Gent Cholesterol and Triglyceride Tests and the Abbott Discrete Analyzer. Means +/- S.E.M. of the four dietary groups were obtained at necropsy.

### Table 33: Serum Total Cholesterol (mg/dL)

Sex (°) was determined to be statistically significant, p=0.0487. Group means not sharing the same superscripts are significantly different.

### Table 34: Serum HDL Cholesterol (mg/dL)

Serum High Density Lipoprotein (HDL) had a slight group (*) statistical significance, p=0.0579; however, upon application of the post hoc test significances were elucidated. Means not sharing the same superscripts are significantly different.

### Table 35: Serum Triglycerides (mg/dL)

No significances, on the basis of group or sex, were found.

### Table 36: Ratio of Serum Triglycerides to Serum Total Cholesterol

No significances, on the basis of group or sex, were found.

Table 33:

| | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | mean | 134 | 141 *a | 129 | 163 |
| | S.E.M. | 7 | 16 | 18 | 7 |
| Females | | 159 | 189 *b | 158 | 153 |
| | | 24 | 22 | 16 | 7 |

Table 34:

| | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | mean | 30.5 *a | 29.1 *ab | 20.6 *b | 26.2 *ab |
| | S.E.M. | 1.0 | 1.4 | 0.9 | 3.2 |
| Females | | 28.5 *ab | 30.3 *a | 25.5 *ab | 28.9 *ab |
| | | 1.7 | 3.8 | 3.6 | 3.4 |

Table 35:

| | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | mean | 43.0 | 48.1 | 44.1 | 60.4 |
| | S.E.M. | 8.7 | 9.4 | 9.8 | 13.6 |
| Females | | 55.3 | 70.4 | 39.8 | 49.0 |
| | | 13.1 | 5.6 | 5.7 | 7.1 |

Table 36:

| | | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|---|
| Males | mean | 0.319 | 0.340 | 0.337 | 0.366 |
| | S.E.M. | 0.056 | 0.063 | 0.058 | 0.072 |
| Females | | 0.313 | 0.400 | 0.256 | 0.320 |
| | | 0.047 | 0.070 | 0.030 | 0.037 |

n = 40

cholesterol, nor triglycerides nor their ratio demonstrated any diet-related significances in this study.

## Discussion

## i: Effect of Dietary (n-3) on Fatty Acid Composition of Erythrocytes

Dietary long chain (n-3) fatty acids supplied with equal amounts of (n-6) had an impact on red cell fatty acid profiles. When a shorter chain (n-3) was provided, a constant ratio of (n-6) to (n-3) of 2.4 was maintained, despite an ample supply of substrate of both series. The provision of longer chain (n-3) reduced this ratio to 0.39. Alterations in the ratio of total (n-6) to total (n-3) reflected diet-induced changes of component fatty acids.

a) Effect on (n-6) Fatty Acids

The results of the analysis of LA (Table 6) indicated a one-third increase in its red cell content when dietary fat was elevated in (n-6) fatty acids. This was only noted in the males fed an (n-6)-rich diet, provided as LA, and required 15 weeks. The group which was provided with both LA and LNA in equal amounts, with half the LA content of the (n-6)-rich diet, showed no change. Despite the failure of LNA to displace LA, longer chain (n-3) fatty acids proved to be antagonists of its incorporation. Groups fed menhaden oil and also provided with equal amounts of (n-3) and (n-6), demonstrated a reduction of LA to one-half its original levels, in 1 to 4 weeks. These findings are consistent with findings in other species, in rats (Monsen et al., 1962) and in marmosets (McMurchie et al., 1986). Compared to fish oil studies in which a change in red cell LA content was indicated, lesser decreases of -13 to -21% were incurred (Sanders et al., 1981; Lorenz et al., 1983; Cartwright et al., 1985; Popp-Snijders et al., 1984, 1986a). In several studies, LA has been noted as the sole or major (n-6) to be displaced (Sanders and Younger, 1981; Iritani and Fujikawa, 1982; Lorenz et al., 1983;

Cartwright et al., 1985). It is interesting to note how rapidly LA content was depleted from erythrocytes compared to how slowly it was enriched.

Similar diet-induced patterns were incurred for AA (Table 7). Males fed a diet rich in (n-6) fatty acids demonstrated a one-third increase in the content of AA over a fifteen week period. The group fed LA and LNA showed no significant effects, whereby groups fed LA and longer chain (n-3) exhibited large decreases of two-thirds in females. Due to the magnitude of the increase of AA in males fed an (n-6)-rich diet and since it had to be derived from LA, this could be taken as evidence of an even larger LA uptake than previously assumed, whereby a portion is converted by delta-6 and -5 desaturases to AA. To the contrary, McMurchie et al. (1986) observed that, in marmosets, AA levels decreased subsequent to dietary and tissue increases in LA. Monsen et al. (1962), Walker and Kummerow (1963) and Housley et al. (1986) noted a resistance of AA to dietary manipulation, in rats and rabbits; which may be a species-specific phenomenon irrelevant to primate species. Likewise, inhibition of conversion of LA to longer chain metabolites, as ascertained by Brenner (1969) in rats, is questioned. The results for the group fed LSO also disputed results found in rats by Weiner and Sprecher (1984); LNA-feeding did not affect cellular arachidonate levels. The incremental decrease of AA over LA for females fed menhaden oil could be interpreted as long chain (n-3) antagonizing not only the esterification of LA but the desaturation and esterification of its metabolite. Other fish oil studies have indicated irregularities in AA manipulation; in those studies where AA did decrease, it was -8 to -12% (Sanders et al., 1981;

Popp-Snijders et al., 1986a). Unlike another study using menhaden oil, the cell content of LA did not accumulate because of competitive inhibition of its desaturation to AA by (n-3) fatty acids (de Schrijver and Privett, 1982). In addition to the demonstration of dietary manipulation of AA in a primate species, it was also shown that the time for and magnitude of accretion of this fatty acid exceeded depletion.

In the case of 22:4, males fed high amounts of LA showed a similar increase in this (n-6) fatty acid, in the absence of (n-3) antagonists (Table 8). Since it was only provided by its precursor, this constituted evidence of conversion of LA to adrenic acid. When LNA was provided, levels appeared to diminish but were not significant. Groups fed the fish oil, previously shown to antagonize precursor (n-6) fatty acids, showed the greatest trend of attenuation of 22:4. A decrease in 22:4 (n-6) induced by long chain (n-3) fatty acids was noted in total phospholipids (Sanders et al., 1981; Cartwright et al., 1985) and in PE fraction (Popp-Snijders et al. 1986a,b) but not in others (Lorenz et al., 1983; von Schacky et al., 1985a).

The red cell 22:5 (n-6) showed a significant and rapid decrease with time, in a group fed an (n-6)-rich diet (Table 9). This same group of females, however, had not previously shown any effects of diet on precursors of this fatty acid nor in (n-3) content. Since it was not provided directly in the diet, it had to be derived from LA, the sole source of (n-6) fatty acids. It was interesting to note that cells supplied with high amounts of LA would have exhibited a decrease in a long chain (n-6). Their male counterparts, which did show an elevation in precursors, did not have significantly elevated 22:5 (n-6), either.

The lack of dietary impact at this level of the (n-6) pathway is noteworthy; it corresponded to a low activity of delta-4 desaturase, as previously noted in man (Sanders and Naismith, 1979) and in other primates (Fiennes et al., 1973; Neuringer et al., 1984). The work of Weiner and Sprecher (1984) which asserted a decrease of this fatty acid upon feeding (n-3) fatty acids was not contradicted by this study, however levels of 22:5 were not appreciable in either series, presumably as a result of low delta-4 desaturase activity.

By the end of the study, males fed high levels of LA had increased in total erythrocyte (n-6) content by one-third, due to enrichment of (n-6) in the diet and to low amounts of (n-3) fatty acid antagonists (Table 10). While LA and AA normally make comparable contributions to the total (n-6) content of red cells, adrenic acid also participated in the increases in total (n-6). These increases were only found in males fed an (n-6)-rich diet and were incurred in the final weeks of the study. Interestingly, erythrocytes of monkeys fed LSO were found to contain almost three times the total (n-6) contents of those fed menhaden oil, although the (n-6) contents of the diets was equal. The red cell (n-6) content of the animals provided with linseed oil did not demonstrate any significant differences, nor did any individual (n-6) fatty acid. This was surprising since their diet provided LNA, which as an (n-3), should have competed with the (n-6) series for desaturation, elongation and/or esterification (Stubbs and Smith, 1984). The erythrocytes of animals receiving marine (n-3), females in particular, showed a significant decrease approaching two-thirds of total (n-6) fatty acids within 9

weeks[20]. This was in agreement with the findings of Marshall and Johnston (1982) and de Schrijver and Privett (1982) in rats; where the conversion of LA to AA and 22:4 (n-6) was inhibited by dietary long chain (n-3), provided by menhaden oil. This also demonstrated that various (n-3) were not equivalent antagonists of (n-6). When longer chain (n-3) were provided in equal amounts to (n-6); the erythrocyte LA content dropped to half its original level; this may have indicated competition of (n-3)s and (n-6)s for esterification despite a reputed advantage of (n-6) (Iritani and Fujikawa, 1984). The AA levels were further reduced from baseline levels, this may have represented a compounded competition for delta-6 and delta-5 desaturases as well as esterification. Esterification of LA appears equally affected in males and females fed long chain (n-3); desaturation and elongation if diminished in females would have explained the further decreases of AA in females from these groups compared to the males. Because no appreciable accumulation of the (n-6) intermediates 18:3 or 20:3 was detected in this study (results not shown) it is difficult to identify which desaturase was implicated. The time for cell accretion of these fatty acids was slower than depletion; which may explain the two-fold difference of their magnitude.

b) Effect on (n-3) Fatty Acids

Linseed oil led to a tripling of red cell LNA content but no significant impact on other (n-3) nor (n-6) fatty acids (Table 11). Within one week, cellular levels of this fatty acid increased 2.3- to 2.7-fold. The results demonstrated that although LNA is not normally a

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[20]Although males fed menhaden oil without an increment in alpha-tocopherol did not show a significance of time, this was due to the limitations of the statistical testing

major erythrocyte membrane acyl component, it can be manipulated expeditiously by the diet to an appreciable level. Metabolites of this fatty acid, 20:4, 20:5 and 22:5 did demonstrate a minor (insignificant) increasing trend which was consistent with the findings in primates and rabbits (Neuringer et al., 1984; Housley et al., 1986). The lack of conversion of LNA to its metabolites could be due to an equal presence of LA (Sanders and Younger, 1981). However, these authors did observe an increase in metabolites in human plasma when LA and LNA were fed in an equal ratio, providing a dose of 6.5 g LNA/day over 2 weeks. Hence, some of this fatty acid may otherwise, as suggested by Sanders and Naismith (1980), have provided a substrate for beta-oxidation. Contrary to the results of Weiner and Sprecher (1984), the concomitant provision of LA and LNA did not favour the conversion and acylation of LA and its metabolites.

The 20:4 (n-3) was not found to be significantly different for any dietary group, not even in monkeys fed LSO, where its precursor was observed to accumulate (Table 12). This can be interpreted as competition for delta-6 desaturase in the presence of an equal quantity of (n-6); which also maintained a constant parallel (n-6) content in these cells. Examination of the results for 20:4 (n-3) revealed that, for the group fed elevated LA, where minimal quantities of this fatty acid, or its precursors were available, cellular content dwindled. However, all groups receiving (n-3) fatty acids also maintained low levels of 20:4 (n-3). The marginal quantities of this fatty acid in any of the groups are suggestive of its immediate desaturation to 20:5 (n-3).

Levels of EPA in the erythrocytes responded to dietary levels (Table 13). The group fed an (n-6)-rich diet demonstrated decreases to one-tenth and the group fed short chain (n-3) to one-third of original eicosapentaenoate levels. On the contrary, groups fed menhaden oil, had by the end of the study, incorporated up to thrice the EPA content of zero-time. The monkeys fed an (n-6)-rich diet exhibited both diminished red cell LNA and EPA contents. Neither of these decreases had reached the 1% level of significance during the study but may have over a more extended period. This group, therefore, demonstrated that unless (n-3) fatty acids are constantly provided by the diet, cellular levels diminish, in favour of the more plentiful (n-6). The results from dietary LSO were provocative. Although LNA was amply incorporated into these cells, its elongated and desaturated metabolites were not. Despite equivalent provision of (n-3), red cells from animals fed LSO contained significantly less EPA than animals fed menhaden oil, within four weeks. EPA levels for this group were not significantly different from those levels in the low (n-3) group, at any time during the study. Because EPA did not increase in these cells, the processes of elongation/desaturation by the liver and esterification by the erythrocytes were questioned. Groups fed menhaden oil, however, provided equivocal evidence that EPA was easily incorporated into these cells; therefore delta-6 and delta-5 desaturation were potentially implicated. Previously, in the group fed an (n-6)-rich diet, adequate desaturase activity was demonstrated for the conversion of LA to AA in the relative absence of (n-3) competitive inhibitors. However, in those fed menhaden oil, this same desaturation/elongation of LA to AA was diminished, as was seen by the

even greater decrease in AA compared to LA levels. This evidence demonstrated that when (n-3) and (n-6) are provided in equal amounts in the diet, desaturation/elongation processes were mutually inhibited in addition to the diminished acylation of precursors. Dietary LSO was also found to have little impact on platelet EPA levels compared to menhaden oil which provided an equal amount of (n-3), but gave more profound effects (Sanders and Roshanai, 1983). Nevertheless, in platelets, the conversion of LNA to EPA was found. Previously, Sanders and Younger (1981) had noted a greater effect of MaxEPA compared to linseed oil on plasma and platelet EPA and DHA levels. Marine-type (n-3) fatty acids inhibited desaturation/elongation of the (n-6) series, but in this instance, there was no competition for the simultaneous desaturation/elongation of the (n-3) series. A corollary of this would be that if AA and other (n-6) metabolites must be derived via desaturation and elongation from LA whereas EPA in its preformed state is readily available; EPA would be incorporated into the cell.

It is noteworthy how relatively rapidly and to which magnitude this essential fatty acid was incorporated. Within 4 weeks, dietary EPA induced significant increases in red cell EPA. Changes of this magnitude, in human volunteers, were found by Terano et al (1983) in 4 weeks, by Cartwright et al. (1985) in 3 to 6 weeks and by Sanders et al. (1981) in 6 weeks. In this respect, the Cynomolgus monkey appeared to be a suitable model for comparison to humans. In the study of Cartwright et al. (1985) however, EPA levels had maximized in three to six weeks with no further increases in total EPA content. In this study, no such erythrocyte maximum was established.

The 22:5 (n-3) did not indicate any significant variations, for any dietary group, during the 15 weeks of study (Table 14). In only a few of the other studies have the levels of this fatty acid increased. Sanders et al. (1981) found an increase of 24%, while Popp-Snijders (1986a) found a 26% increase. Because ample EPA was provided by menhaden oil; because of a decrease in parallel (n-6) metabolism and because of an apparent low delta-4 activity; one would have anticipated that 22:5 (n-3) might have accumulated in these cells, as was observed in platelets by Sanders and Roshanai (1983). Instead, the red cell levels of this fatty acid in groups fed long chain (n-3) were no different from other groups, whose diets did not contain 22:5 (n-3). This might be interpreted as a haemostatic mechanism controlling the amount of 22-carbon (n-3) fatty acids. Horrobin (1983) noted the consistency of red cell C22 content.

The DHA results indicated a rapid incorporation of this fatty acid into the red cell, after at least 4 weeks of dietary intake (Table 15). The incorporation of DHA observed in this study for animals fed menhaden oil was generally greater in magnitude than Sanders et al. (1981), Lorenz et al. (1983), Terano et al. (1983), Cartwright et al. (1985) and Popp-Snijders et al. (1986a), studies which spanned 3 to 8 weeks. The results were similar to Cartwright et al. (1985) for PC in 3 to 6 weeks but less than the 100% increase found by Popp-Snijders et al. (1984) in 2 weeks. As with EPA, one group of females demonstrated the greatest increase. By the end of the study, this group had almost doubled in DHA content while the group fed menhaden oil and elevated alpha-tocopherol increased up to 77%. The male counterparts, fed menhaden oil without elevated tocopherol, merely showed a 29% increase, which is approximately one-

third of the increase in the females. Monkeys fed either high LA or LA and LNA showed decreases in DHA levels as time progressed, these changes may have become significant upon prolongation of the study. By the end of the study, groups fed long chain (n-3) red cells contained a 3-fold increment in DHA versus the other groups. Enrichment of LNA did not result in any more DHA than those receiving LA alone, which has also been found in plasma and platelets (Sanders and Younger, 1981; Sanders and Roshanai, 1983). These findings also corroborated those of Terano et al. (1983) who did not observe an increase in DHA levels upon feeding purified EPA to humans. These findings were taken as evidence of a lack of its biosynthesis from EPA. The delta-4 desaturase involved in the final steps of conversion of LNA to DHA was previously shown to have low activity in the (n-6) series as well as in man (Sanders and Naismith, 1979) and other primates (Fiennes et al., 1973; Neuringer et al., 1984).

The cellular content of DHA compared to EPA can be explained by their relative dietary proportions, in either case, direct dietary intake was apparently responsible for the cellular levels. An additional explanation for the lesser levels of DHA was its relatively slower incorporation. Significantly increased levels were observed for EPA within 4 weeks, however DHA incorporation took 4 to 15 weeks. This may have pertained to the location of this fatty acid in the red cell or its ability to displace (n-6). However, examination of the results of Cartwright et al. (1985) did not indicate a particular affinity for any phospholipid species of the outer or the inner monolayer. The slower increase in red cell DHA levels may instead have reflected a subsequent selective acylation by other tissues, such as the brain or retina, where

it is a vital structural component (Tinoco et al., 1979). Nevertheless, groups fed menhaden oil have demonstrated that the red cell contents of EPA and DHA are susceptible to dietary manipulation in the time-frame suggested by Farquhar and Ahrens (1963). These results were compatible to those of Sanders and Younger (1981) and Sanders and Roshanai (1983) for plasma and platelets. At zero-time, the red cell content of DHA exceeded that of EPA by up to 53%, at the end of the study, these cellular levels had become equal; where EPA increased up to 219% and DHA increased up to 93%. Several (n-3) studies have exclusively discussed the ramifications of EPA incorporation; failing to recognize the simultaneous impact on cellular DHA levels and their consequences (Sanders, 1985). This area requires further study since these fatty acids do not possess identical physiological roles.

Upon dietary enrichment, the total red cell (n-3) content significantly increased in 4 weeks, coinciding with EPA incorporation (Table 16). By the end of the study, groups fed long chain (n-3) contained up to a doubled (n-3) content; which was 2.3-fold the (n-3) content of the group fed shorter chain (n-3) and 4.6-fold the group fed (n-6). The group fed the (n-6)-rich diet, indicated a depletion of total (n-3) content of up to 62%, which had not reached the 1% level of significance by the end of the study. This depletion was not attributable to any one (n-3) fatty acid, nor was there evidence of retention of a specific (n-3), although small amounts were provided as LNA. A similar depletion was found in marmosets (McMurchie et al., 1986). The level of (n-3) for monkeys fed LA corresponded to half that of those fed LA and LNA, reflecting the antagonism of (n-3) for (n-6).

Despite the large increment in its LNA content, the group provided with LSO did not demonstrate any impact on its (n-3) derivatives nor in total (n-3). This signified the minor impact of dietary LNA on the red cell. In the absence of marine fatty acids, 22:5 was the major (n-3) for females in this group. One should note the large difference in total (n-3) incorporation for one group of females compared to other groups fed menhaden oil. The displacement of (n-6) fatty acids for all groups was similar but the extraordinary incorporation of EPA and DHA in that group of females contributed to differences in total (n-3).

It was interesting to note that the depletion of (n-6) was not reciprocally related to (n-3); (n-6) decreased approximately 60% but the accretion of (n-3) was approximately 80%. This discrepancy, also noted by von Schacky et al. (1985a), may have pertained to slight, but insignificant, adjustments in non-polyunsaturated fatty acids; since the total polyunsaturate levels remained at 50% (results not shown). As previously noted, each phospholipid species has a characteristic fatty acid affinity with respect to saturated and unsaturated fatty acids (Ways and Hanahan, 1964; Williams et al., 1966; Dodge and Phillips, 1967). Tolerance to changes in the lipids occupying the 2-position is limited to species which can maintain optimal configuration for lipid/lipid and lipid/protein interactions in the membrane (Kuypers, Roelofsen, Op den Kamp and van Deenen, 1984). It is therefore not coincidental that other fatty acids are readily incorporated into the 1-position subsequent to changes at the 2-position fatty acyl components (Op den Kamp, Roelofsen and van Deenen, 1985). Additionally, there was a higher absolute content of (n-6) fatty acids in the red cell compared to (n-3); therefore a

disproportionate exchange, on a percentage basis, would be required. The results of total (n-3) fatty acids showed that the addition of long chain (n-3) to the diet had an impact on red cell fatty acid profiles, despite the provision of equal amounts of (n-6) fatty acids. In other tissues, this substitution would have been associated with a lowered substrate availability and competitively inhibited production of 2-series eicosanoids, in favour of the 3-series.

c) Effect on the Ratio of Total (n-6) to Total (n-3) Fatty Acids

With the use of the ratio as total (n-6) to total (n-3) only the high (n-6) group was significant from the others (Table 17). The greatly elevated (n-6)/(n-3) ratio in that diet corresponded to changes in the erythrocyte fatty acids. The lapse in time before intra-group significances of the ratio were reached indicated that RBC were an index of long-term dietary intake, requiring the time for turnover of the cellular fatty acids.

A plot of (n-6) to (n-3), in Figure 1, also depicted the effect of the (n-3) diets on this ratio throughout the study, illustrating cellular control via acyltransferases and desaturases. When the diet supplied similar amounts of LA and LNA, as 18-carbon substrates, the ratio of total (n-6) to total (n-3) remained constant. This indicated a close regulation of the conversion to long chain fatty acids and their incorporation into the cell. One might have anticipated antagonism of (n-3) fatty acids for (n-6) and a decreasing tendency in their ratio. However, the ratio of 2.4 was maintained. One must question the relevance of a ratio of 2.4, whether it represented an optimal proportion of these essential fatty acids or whether it represented the original

proportions established at erythropoiesis. This plot demonstrated acylation was closely regulated, obviously favouring (n-6) fatty acids. Desaturases, known to favour (n-3), were demonstrated to be inhibited by the dietary ratio, since long chain metabolites of LNA did not accumulate (Brenner, 1966).

A supply of preformed C20- and 22-(n-3) fatty acid substrates permitted an enhanced concentration of them in membrane lipids. Less metabolic regulation may be involved by the elimination of desaturation and elongation of substrates. Long chain (n-3) provided by fish oil replaced (n-6) provided as their 18-carbon substrate, producing a ratio of 0.39 (Figure 1). This is one-sixth of the ratio noted at the onset of the study and seen to be maintained in the shorter chain (n-3) group. Since the incorporation of longer chain (n-3) deviates from the cell norm, while shorter chain (n-3) maintained the norm; it provided evidence that the process of desaturation was a cellular control mechanism. The ability to circumvent normal control mechanisms resulted in an extraordinary incorporation of (n-3) fatty acids in erythrocytes. Compared to those fed a plant-derived source of (n-3), those fed an equal amount of fish-derived (n-3) fatty acids incorporated twice as much (n-3) and were inherently more potent in displacing (n-6) fatty acids. The profile of the curve is asymptotic, indicating changes in this ratio had plateaued. The ratio of 0.39 may therefore have represented a minimum critical content of (n-6) fatty acids or perhaps a maximal (n-3) content in these cells. This might be further elucidated by additional studies where (n-6) and (n-3) fatty acids are provided in varied ratios. Cartwright et al. (1985) noted a maximal total EPA content of

erythrocytes (163% original) which subsequently underwent redistribution among erythrocyte phospholipids.

On the other hand, the group fed an (n-6)-rich diet showed no signs of a plateau or a maximum ratio (Figure 1). The constant increase in their ratio was attributable to both an increase in (n-6) and decrease in (n-3) fatty acids. This trend was slightly more pronounced in the females despite males having increased significantly in (n-6) fatty acids. The plot for these monkeys demonstrated the affinity red cell acyltransferases have for (n-6) fatty acids upon dietary enrichment. By end of the study, the ratio had reached 6.6, nearly three-fold its original value. This curve may never have plateaued since the numerator was increasing and the denominator was approaching zero, overall the system approached infinity. By the same rationale, groups fed long chain (n-3), with the inverse situation, would have approached zero over time.

d) Effect of Sex on Fatty Acid Composition

One aspect of these results was the relatively stronger response of females versus males to the diet, particularly in the lack of (n-6) accretion and in the incorporation of (n-3) short and long chain fatty acids (Table 18). When expressed as the summation of (n-6) versus (n-3) these differences were somewhat sublimated and did not reach the 1% level of significance. These differences cannot be related to discrepancies in dietary intake (Tables 31 and 32). Hagve and Christophersen (1986) had previously noted sex-related differences in metabolism of essential fatty acids in isolated rat liver cells. Female rats exhibited elevated triacylglycerol synthesis and lower oxidation and phospholipid biosynthesis rates compared to males. Only minor gender differences were

found in the 22-carbon polyenoic series. Pudelkewicz, Seufert and Holman (1968) have also found sex-related differences in the degree of unsaturation in the liver, heart, erythrocytes and plasma lipids in rats. The female double bond indices were 1.3- to 1.6-times that of the males a finding which was taken to indicate that chain elongation and desaturation occur more readily in females. One must note that these enzymes are known to favour (n-3) fatty acids and are subject to hormonal stimuli (de Schrijver and Privett, 1982); evidence of both appeared in this study. In humans, Holman, Smythe and Johnson (1979), failed to find any significant sex-related differences for individual serum fatty acids; Bates, van Dam, Horrobin, Morse, Huang and Manku (1985) were unsuccessful when total plasma phospholipids were studied.

e) Effect on Non-polyunsaturated Fatty Acids

The analysis of total monounsaturated (including 18:1) and total saturated fatty acids (including 16:0, 18:0, 20:0) revealed no significant diet-related differences (Tables 19 to 24). The parameter under study involved the 2-position of glycerophosphatides, rather than the 1-position, where these fatty acids are predominantly located (Lorenz, 1983). Because of the protocol followed, it would have been surprising if any diet-induced changes occurred in these totals, albeit individual fatty acids may well have varied. Significances, however, were incurred with time alone for some of these fatty acids. The effect of time cannot be attributed to the performance of the gas chromatograph since an internal standard was employed. The effect of such diets on monounsaturates and saturates of other tissues also supplied by plasma, the time-frame and their requirements, subsequent to substitution at the

2-position, is not well understood, nor are the repercussions on the red cells.

f) Effect of Alpha-Tocopherol on Fatty Acid Composition

No disparate results were encountered between the groups fed menhaden oil, the addition of tocopherol had no effect on the composition of erythrocyte fatty acids, as seen previously (Fitch et al., 1961; Walker and Kummerow, 1964a; Buckingham, 1985).

g) Effect of Time on Fatty Acid Composition

Time-course analysis was another component of this study. The 4 to 6 week delay (Farquhar and Ahrens, 1963) for the diet to have an impact on human erythrocyte composition was ratified in this study by the majority of the fatty acids. However, with a halved half-life of monkey erythrocytes (Greenberg and Moon, 1961) and a 10-fold acceleration of circulation in primates (Malinow, 1979), one might have anticipated a faster impact of diet and a decelerated incorporation by the end of 15 weeks. Although erythrocytes reacted slowly to dietary manipulation, LNA was an exception, doubling and tripling its content in the first week. This rate exceeded normal cell turnover and indicated a rapid equilibration with plasma. Increases of EPA and DHA required more time as did the corresponding decrease of (n-6) fatty acids. A lag in incorporation has previously been ascribed to confines established at erythropoiesis; perhaps the cell can accommodate LNA easily. The (n-3) in the linseed oil diet was provided exclusively as LNA whereby in the menhaden oil diets several fatty acids contributed to the total (n-3) content; hence the differences in their incorporation may also have pertained to dietary proportions. The fatty acyl composition of the 2-

position was subject to more than dietary provision and the processes of desaturation and elongation. Longer chain (n-3) have been demonstrated to possess essential fatty acid properties in other tissues and for eicosanoid biosynthesis. The specificities of these other tissues, supplied by the plasma, may have been responsible for the slower accumulation of the longer chain (n-3) fatty acids in the erythrocyte.

## ii: Effect of Dietary (n-3) Fatty Acids on Other Haematological Parameters

Because the dietary manipulation of the fatty acid composition in the erythrocyte could have made them more labile to peroxidation, the effects of increased tocopherol and peroxidative tests were evaluated.

a) Effect on Superoxide Dismutase Activity in Erythrocytes

The results of the SOD assays were perplexing, only time was revealed as a significant factor (Table 25). Terminal levels of red cell SOD were their lowest; whereas fatty acid incorporation of longer chain (n-6) and (n-3) for this time interval were maximal. It is apparent that SOD did not correlate to the diet-induced changes in erythrocytes in this study.

b) Effect on Glutathione Peroxidase Activity in Erythrocytes

Although females group 2 demonstrated a greater GSH-Px activity than their male counterparts at zero-time; their total (n-6) and (n-3) fatty acid contents were found to be lower (Table 26). Both the incorporation of fatty acids (LA, EPA and DHA) and the statistical results of the orthogonal decomposition of GSH-Px gave credence to an induction of this enzyme at week 4, when a significant elevated activity was found.

c) Effect on Hepatic Enzyme Assays

Comparison of erythrocyte peroxidative enzyme results was made to those of liver tissue assayed at necropsy (Svend Kaasgaard, thesis in preparation). Hepatic SOD also did not exhibit group-related significances. A trend, however, was observed for sex-related differences, where females showed greater activity. In the fatty acid analyses, females were associated with elevated (n-3) incorporation. Liver GSH-Px activity for the group fed an (n-6)-rich diet, particularly females, was three-quarters that of a group fed menhaden oil. This menhaden oil group was observed to incorporate long chain (n-3) fatty acids in the absence of an increment of tocopherol, its levels were also slightly (but insignificantly) greater than both other groups fed (n-3) fatty acids; perhaps these findings might have been related to the diet. Catalase activity, assayed in the liver only, demonstrated sex-related differences where females in groups fed LA or LA and LNA were significantly greater than males. No aspects of fatty acid incorporation into the red cells could be reconciled with these results.

d) Effect on Thiobarbituric Acid Assays of Whole Blood

MDA, measured by the TBA assay, can be an index of membrane peroxidative damage; it has been correlated to a disturbed phospholipid asymmetry and hypercoagulability in erythrocytes (Jain, 1985). Previous attempts to demonstrate an increased susceptibility to peroxidation using TBA were unsuccessful (Walker and Kummerow, 1964a). In the present study, significant differences among groups occurred by the fourth week; where females on two of the (n-3) diets had elevated TBA values compared to group on the (n-6) diet and to themselves at other time intervals

(Table 27). These elevated TBA values could have been indicative of an increased peroxidation or an altered eicosanoid synthesis. Week 4 was previously associated with elevated erythrocyte GSH-Px activity and initial incorporation of (n-6) and (n-3) fatty acids. It is possible that the introduction of triene fatty acids did have an impact on cell peroxidation. The results of the remaining (n-3) dietary group and the orthogonal decomposition did not contradict these findings. However, none of these assays provided conclusive evidence of diet-induced peroxidative stress in the red cell, nor was there evidence that surplus tocopherol was required.

e) Effect on Platelet Cell Counts

The thrombocytopenia previously noted in the literature associated with large doses of (n-3)(Hay et al., 1982) was not observed in this study (Table 28). Instead, a generalized decrease including the (n-6)-enriched group, was observed. One would have expected that a decrease in platelet counts would have been associated with a concomitant increase in bleeding time and bleeding time blood volumes; which was also not found in this study.

f) Effect on Bleeding Time

An increase in BT was anticipated upon (n-3) feeding (Sanders et al., 1981; Harris et al., 1983; Lorenz et al., 1983; Sanders and Roshanai, 1983; Thorngren et al., 1983; Ahmed and Holub, 1984; Fischer and Weber, 1984; Saynor and Verel, 1984). One might also have anticipated that a high linoleate diet would result in a group significance because of its enrichment in (n-6) fatty acids, including AA, which would be associated with a proaggregatory state in platelets.

However, the generalized decrease in BT after week 6 directly contradicted the enhanced incorporation of (n-3) into erythrocytes and failed to distinguish the incorporation of (n-6) fatty acids in erythrocytes of one dietary group (Table 29).

g) Effect on Bleeding Time Blood Volume

BTBV, which showed a reduction, was also contrary to the previous findings (Thorngren et al., 1983c). Neither an (n-3)-induced increase nor an (n-6)-related decrease was distinguishable (Table 30). The maximum BT did coincide with the maximum BTBV, as seen previously (Thorngren et al., 1983a,c).

Haematological properties of these monkeys were inconsistent with reports in the literature despite ample time for such alterations as well as alterations in platelet fatty acid composition to occur. In this instance, these findings may result from the equal provision of (n-6) fatty acids; rather than a unilateral increase in (n-3) fatty acids. These and previous inconsistencies in haematological properties led to the conclusion that the beneficial effects of (n-3) fatty acids pertain to their role as membrane structural components.

h) Effect on Serum Cholesterol and Triglyceride Levels

Mueller, Schiefer, Laxdal and Ackman (1982) previously found Cynomolgus monkey total cholesterol concentrations of 130-150 mg/dL; which was compatible with the present findings (Table 33). These authors also observed that serum cholesterol in Cynos responded readily to dietary manipulations. The findings of Harris et al. (1983) of an increased hypocholesterolemic or hypotriglyceridemic effect of (n-3) fatty acids was not evident in this study; groups fed dietary (n-3) fatty

acids were not significantly different than the group fed high linoleate (Tables 33 to 36). An impact of dietary (n-3) fatty acids on plasma lipoproteins and triglycerides is dependent on both the source and the dose; previously only studies utilizing elevated doses of short and long chain (n-3) have observed a decrease in VLDL, LDL, total cholesterol and triglycerides (Harris et al., 1983; Illingworth et al., 1984; Simons et al., 1985; Phillipson et al., 1985; Nestel, 1986) whereby HDL is increased (Saynor and Verel, 1980, 1984; Sanders et al., 1981; Sanders and Hochland, 1983; Sanders and Roshanai, 1983; Nestel, 1986). Moderate doses have previously been associated with a lack of effect on cholesterol (Thorngren et al., 1986). It is probable that the nature of these diets, in which the level of polyunsaturates was kept constant and in which (n-3) fatty acids were balanced by an equal amount of (n-6)-linoleate, may be responsible for the lack of dietary impact on the serum lipids studied, despite those changes observed in erythrocytes.

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# Appendix

## List of Abbreviations

| | |
|---|---|
| AA | arachidonic acid |
| BT | bleeding time |
| BTBV | bleeding time blood volume |
| C:P | cholesterol:phospholipid |
| C20-22 | 20-22 carbon atoms |
| CHD | coronary heart disease |
| CLO | cod liver oil |
| Cyno | Cynomolgus |
| DBI | double bond index |
| DGLA | dihomo-gamma-linolenic acid (20:3, n-6) |
| DHA | docosahexaenoic acid (22:6, n-3) |
| EFA | essential fatty acid |
| EPA | eicosapentaenoic acid (20:5, n-3) |
| GSSG | oxidized glutathione |
| GSH-Px | glutathione peroxidase |
| HDL | high density lipoprotein |
| kcal | kilocalories |
| LA | linoleic acid (18:2, n-6) |
| LDL | low density lipoprotein |
| LNA | (alpha) linolenic acid (18:3, n-3) |
| LSO | linseed oil |
| MDA | malondialdehyde |
| NADP | B-nicotinamide adenine dinucleotide |
| NADPH | B-nicotinamide adenine dinucleotide (reduced) |
| N.S. | not significant |
| $^{\bullet}O_2$ | superoxide radical |
| PC | phosphatidyl choline |
| PE | phosphatidyl ethanolamine |
| PG | prostaglandin |
| PGI | prostacyclin |
| PI | phosphatidyl inositol |
| PL | phospholipid |
| PPP | platelet-poor plasma |
| PRP | platelet-rich plasma |
| PS | phosphatidyl serine |
| P/S | polyunsaturate/saturate |
| PUFA | polyunsaturated fatty acid |
| RBC | red blood cell |
| SD | standard deviation |
| S.E.M. | standard error of the mean |
| SM | sphingomyelin |
| SOD | superoxide dismutase |
| TBA | thiobarbituric acid |
| TBARS | thiobarbituric acid reactive substances |
| TG | triglyceride |
| TX | thromboxane |
| ug | micrograms |
| uL | microliters |
| VLDL | very low density lipoprotein |
| WBC | white blood cell |

## Fatty Acid Nomenclature[1]

| Formula | Systematic Name | Trivial Name |
|---|---|---|
| C12:0 | Dodecanoic | Lauric |
| C14:0 | Tetradecanoic | Myristic |
| C14:1 (n-5) | 9-Tetradecenoic | Myristoleic |
| C16:0 | Hexadecanoic | Palmitic |
| C16:1 (n-7) | 9-Hexadecenoic | Palmitoleic |
| C17:0 | Heptadecanoic | Margaric |
| C18:0 | Octadecanoic | Stearic |
| C18:1 (n-7) | 11-Octadecenoic | Vaccenic |
| C18:1 (n-9) | 9-Octadecenoic | Oleic |
| C18:2 (n-6) | 9,12-Octadecadienoic | Linoleic |
| C18:3 (n-3) | 9,12,15-Octadecatrienoic | alpha-Linolenic |
| C18:3 (n-6) | 6,9,12-Octadecatrienoic | gamma-Linolenic |
| C18:4 (n-3) | 6,9,12,15-Octadecatetraenoic | |
| C19:0 | Nondecanoic | |
| C20:0 | Eicosanoic | Arachidic |
| C20:1 (n-9) | 11-Eicosenoic | Gondoic |
| C20:1 (n-11) | 9-Eicosenoic | Gadoleic |
| C20:2 (n-6) | 11,14-Eicosadienoic | |
| C20:3 (n-6) | 8,11,14-Eicosatrienoic | dihomo-gamma-Linolenic |
| C20:4 (n-6) | 5,8,11,14-Eicosatetraenoic | Arachidonic |
| C20:4 (n-3) | 8,11,14,17-Eicosatetraenoic | |
| C20:5 (n-3) | 5,8,11,14,17-Eicosapentaenoic | EPA |
| C22:0 | Docosanoic | Behenic |
| C22:1 (n-9) | 13-Docosenoic | Erucic |
| C22:1 (n-11) | 11-Docosenoic | Cetoleic |
| C22:4 (n-6) | 7,10,13,16-Docosatetraenoic | Adrenic |
| C22:5 (n-3) | 7,10,13,16,19-Docosapentaenoic | |
| C22:5 (n-6) | 4,7,10,13,16-Docosapentaenoic | |
| C22:6 (n-3) | 4,7,10,13,16,19-Docosahexaenoic | DHA |
| C24:0 | Tetracosancic | Lignoceric |

[1] all in methylene-interrupted cis configuration

## i: Statistical Analyses

The BMDP Statistical Software package (Jennrich, Sampson and Frane, 1985) was employed to determine the analysis of variance (ANOVA) for each assay in the study. Such an analysis was demanded since the data were both repeated and related; secondly, in a subpopulation with large variances, this analysis allowed each animal to serve as its own control.

The BMDP 2V package provided an ANOVA table, which accounted for repeated measures, between-group and within-group factors and variable cell sizes (n).

In repeated measures models, repeated measurements are made on the same variable, for the same subject, at different times and thus cannot be considered statistically independent. In this application, the performance of each monkey was measured repeatedly, to determine the effect on an experimental parameter, over a prolonged time period. This type of statistical evaluation permitted: i) comparison of the overall performance of groups, ii) the evaluation of group performance with respect to time and iii) an indication of the rate of performance changes between time intervals.

With this model, a distinction was made between grouping factors (sex and diet) and within-subjects factors (time interval). Hence, grouping factors referred to 'between group' variances; while 'within-subjects' factors related to within-group variances. Therefore, this application analyzed for two grouping factors and one within-factor; as well as all interactions of these three factors.

Time series analyses are usually based on the assumption that the data represent measurements at equispaced time intervals. A statement

included in this analysis asserted that the within factor of time was not at equally spaced intervals; but represented measurements taken at weeks 0,1,4,9 and 15.

Lastly, the BMDP 2V analysis also provided the orthogonal decomposition of the polynomial components into linear, quadratic, cubic and more complex forms. This delineated trends in the data which can be described by mathematically complex equations for the curve that best fits the data.

## ii: Duncan's Post Hoc Statistical Test

The post hoc statistical test applied to the ANOVA table, in each case, was the Duncan's Multiple Range test. This test takes a layered or stairstep approach to the making of multiple comparisons. Instead of making all pairwise comparisons in relation to a single critical difference, such as in the t-, the Tukey and the Scheffē tests, the size of the critical difference is adjusted depending upon whether the two means being compared are adjacent or whether other means fall between those being compared (Bruning and Kintz, 1977). It has an advantage over the Dunnet's test, in that all possible comparisons can be made; rather than simply the comparison of a test mean against that of a control group.

The basic computational formula for the Duncan's Multiple Range test is (Kirk, 1968):

$$W_r = Q_{r_\alpha;r,v}\sqrt{\frac{MSerror}{n}}$$

$W_r$ = critical difference
$Q_r$ = Duncan's Significant Studentized Ranges, at $\alpha$ level of significance
$r$ = number of means for range being tested
$v$ = degrees of freedom associated with mean square error
MSerror = mean square within group error
$n$ = number of replicates

The first step in the analysis was to rank the means in order of magnitude. $W_r$ was the critical difference of these two ranked means that must be exceeded in order to be declared significant. $Q_r$ was obtained from tables of Duncan's Significant Studentized Ranges, for each value of $r$, $\alpha$ and $v$. The product of $Q_r$ and the square root of MSerror/n was

computed and compared to the critical difference ($W_r$) to determine the significance.

An extension of the Duncan's New Multiple Range test for the case of unequal number of replicates, was described by Kramer (1956), which transformed the Duncan's formula:

$$(\bar{X}_B - \bar{X}_C) > R'_p$$
$$> Sz_p \times Z_{p.n2}$$
$$> \sqrt{\frac{1}{2}\left(\frac{1}{n_B} + \frac{1}{n_C}\right)s^2} \times Z_{p,n2}$$

$$\boxed{(\bar{X}_B - \bar{X}_C)\sqrt{\frac{2n_B \times n_C}{n_B + n_C}} > S_z \times Z_{p.n2}}$$

$\bar{X}$ = group mean
$R'_p$ = critical difference
$Z_{p.n2}$ = Duncan's Significant Studentized Ranges
$S_z$ = standard error of mean $= \sqrt{\frac{1}{2}\left(\frac{1}{n_B} + \frac{1}{n_C}\right)s^2}$
$n_2$ = degrees of freedom error of mean square
$p$ = number of means for range being tested
$s^2$ = mean square for error
$n_i$ = number of replicates for each mean

The Duncan test for unequal ns collapses to a t-test when there are two (adjacent) means being compared.

## Erythrocyte Fatty Acid Composition

Figures 2 through 9 represent the (n-6) family of fatty acids for each diet and each sex, obtained from gas chromatographic analysis of Cynomolgus monkey erythrocytes. Diet 1 contained lard and corn oil (high linoleic acid), Diet 2 contained lard and linseed oil (high linolenic acid) and Diets 3 and 4 contained lard, corn oil and menhaden oil (high long chain n-3). All diets were isocaloric and provided equal total polyunsaturate contents; however diet 1 provided (n-6), diet 2 equal (n-6) to (n-3), diets 3 and 4 equal (n-6) to longer chain (n-3) fatty acids, as the source of polyunsaturates. Diet 4 differed from diet 3 by a four-fold enrichment of alpha-tocopherol.

Figure 2: Plot of Males Group 1 (n-6) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (T) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.

Figure 2

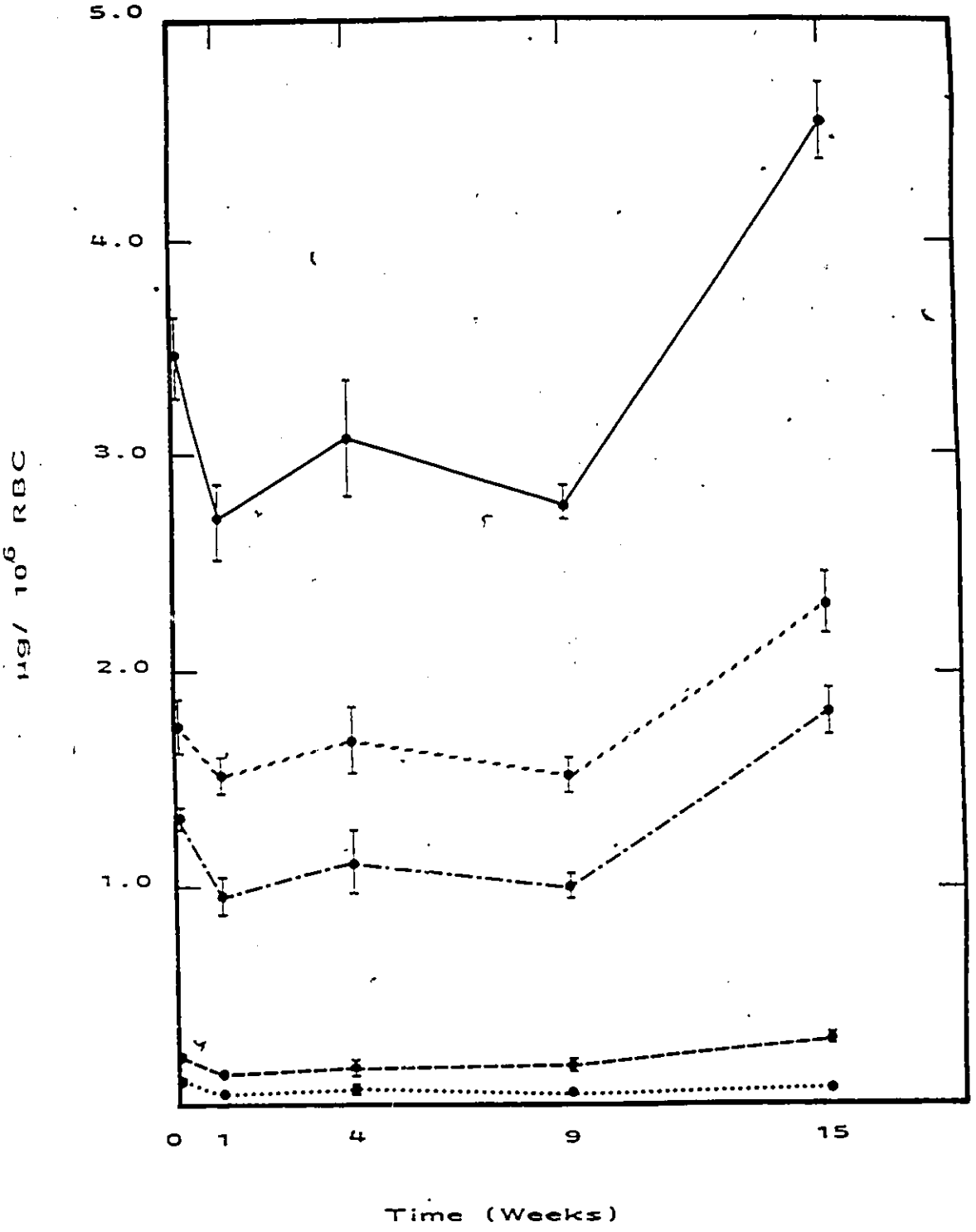


Figure 3: Plot of Females Group 1 (n-6) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group mean +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.

- 18:2
- 20:4
- 22:4
- 22:5
- total (n-6)

Figure 3

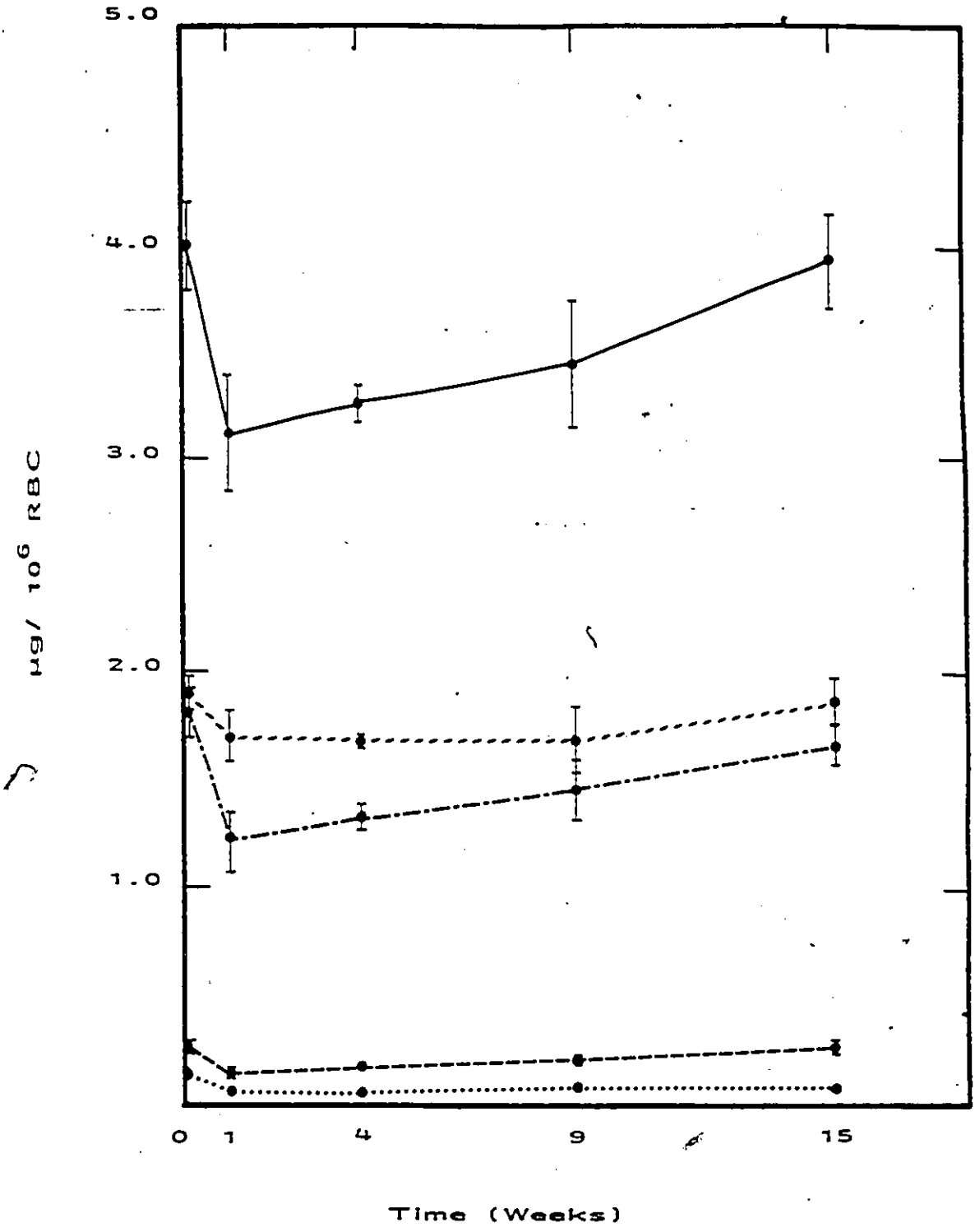


Figure 4: Plot of Males Group 2 (n-6) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.

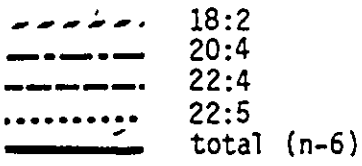


Figure 4

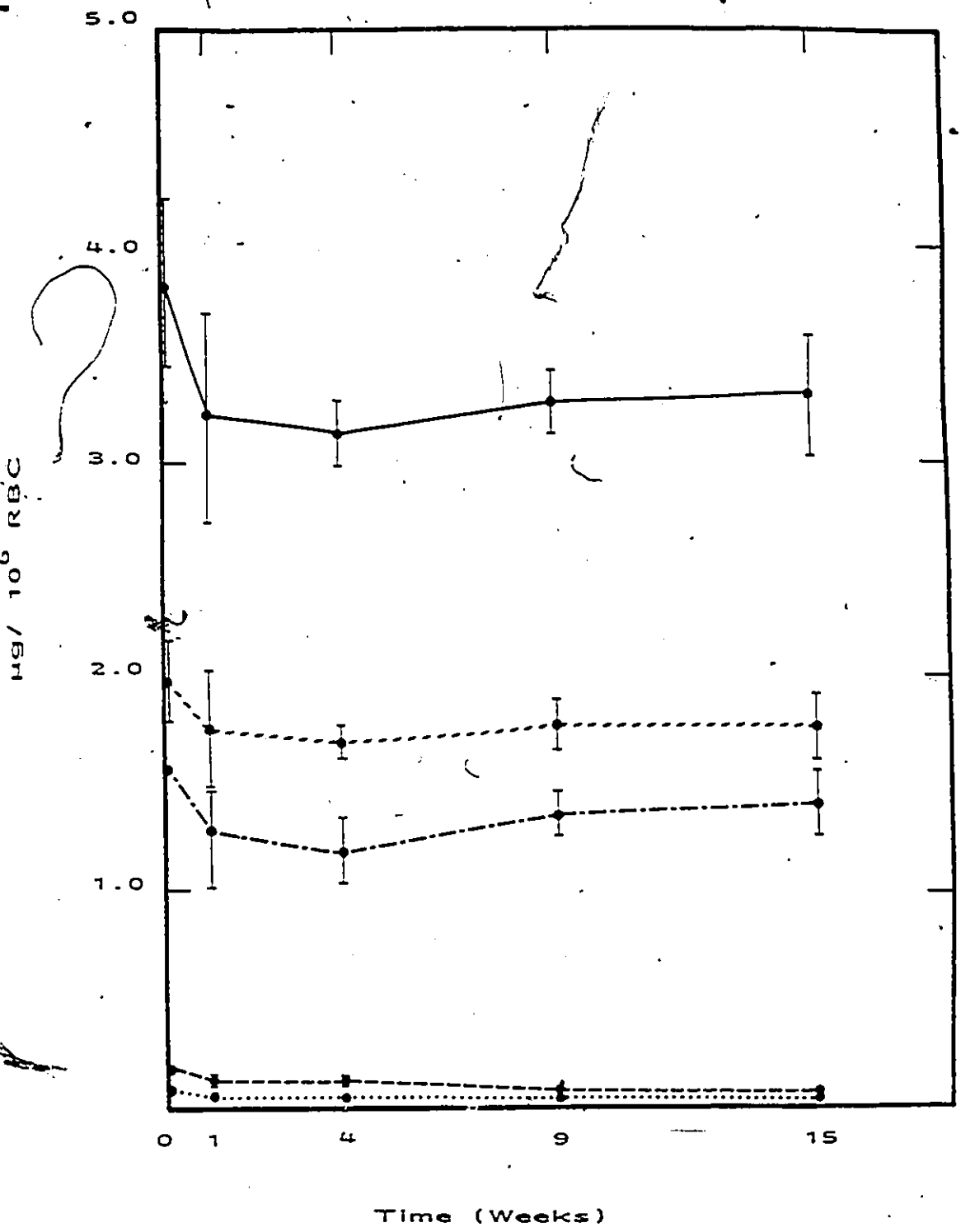


Figure 5: Plot of Females Group 2 (n-6) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.

- 18:2
- 20:4
- 22:4
- 22:5
- total (n-6)

Figure 5

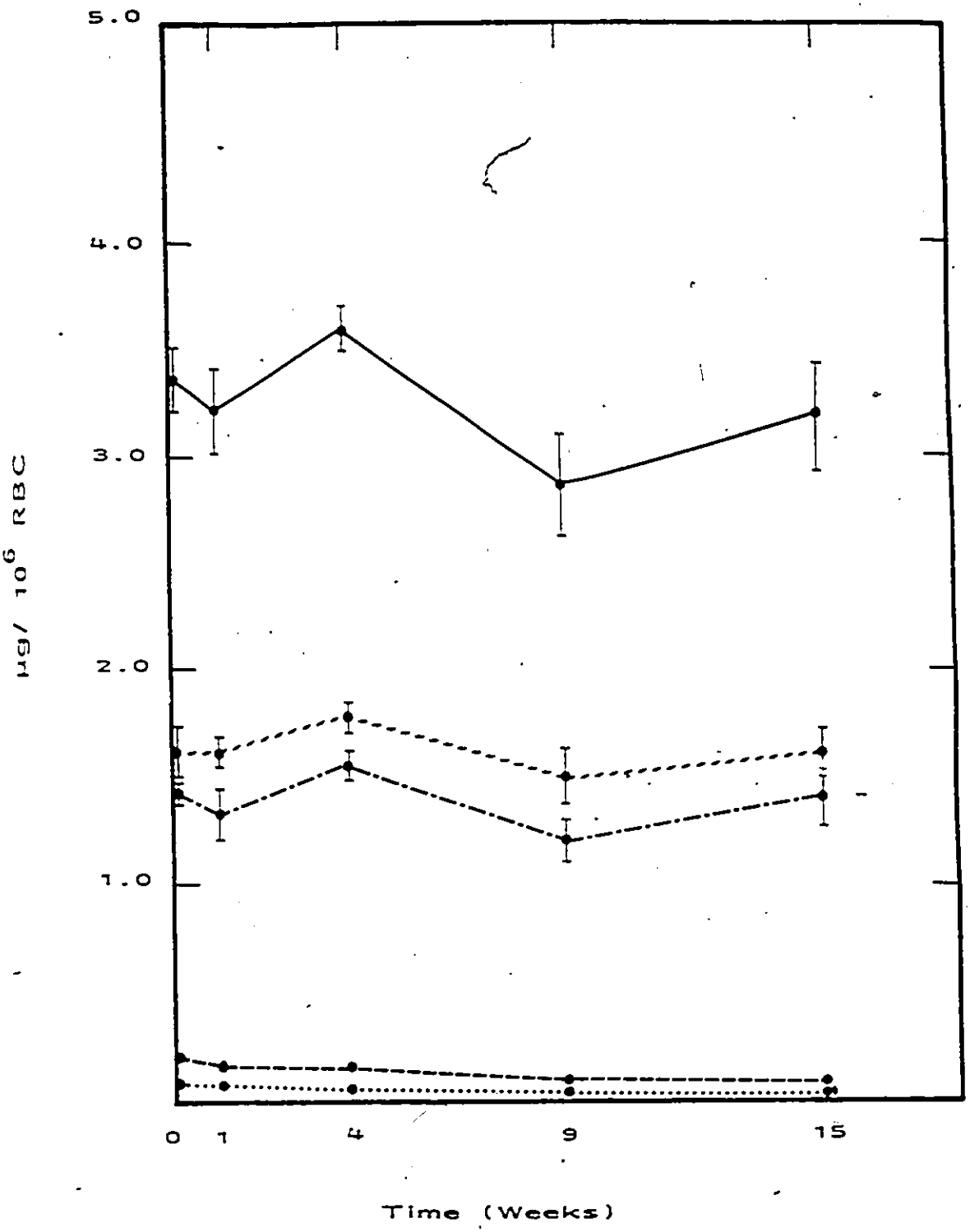
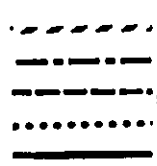


Figure 6: Plot of Males Group 3 (n-6) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.



18:2
20:4
22:4
22:5
total (n-6)

Figure 6

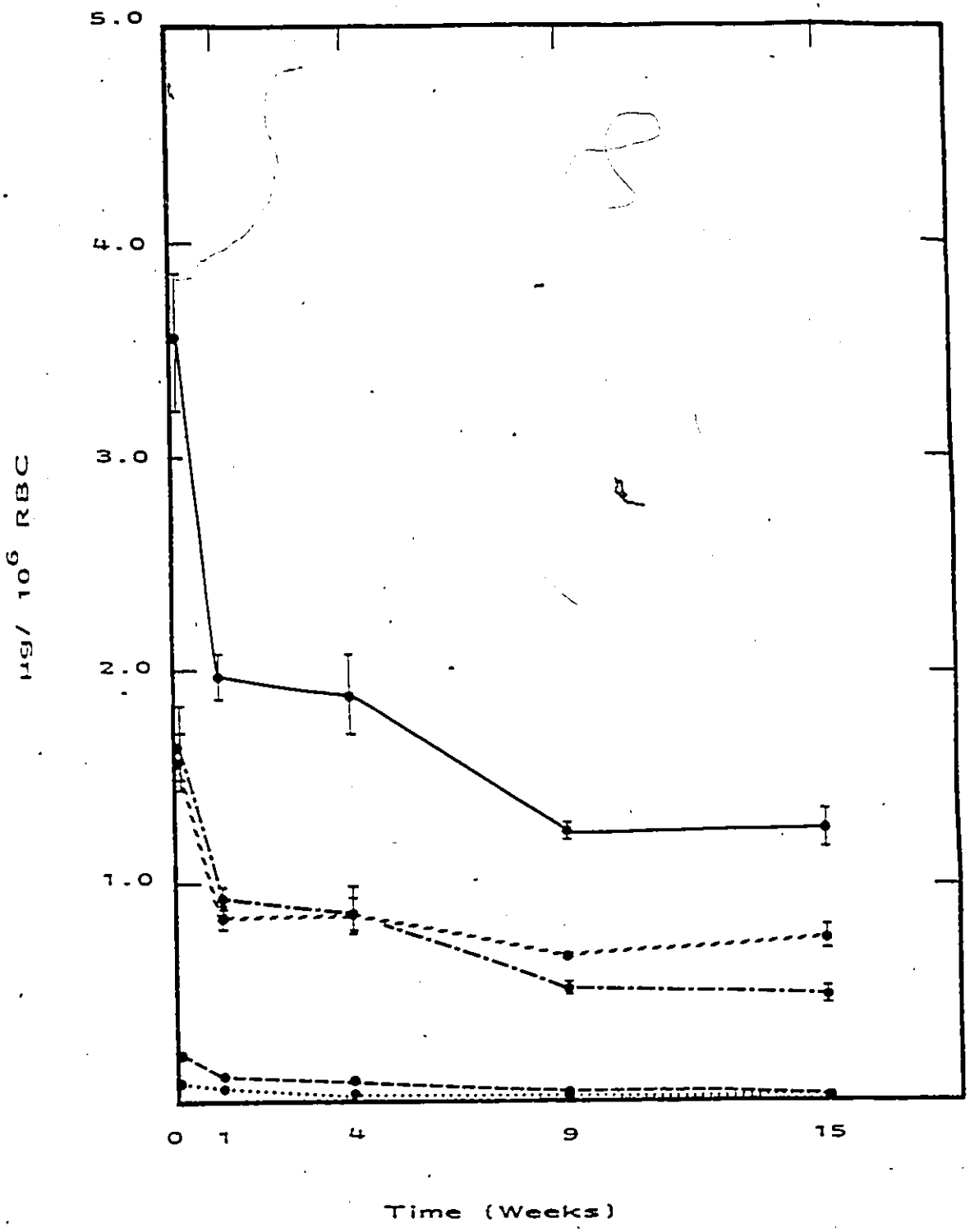
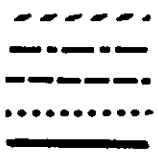


Figure 7: Plot of Females Group 3 (n-6) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time ($\dagger$) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.



18:2
20:4
22:4
22:5
total (n-6)

Figure 7

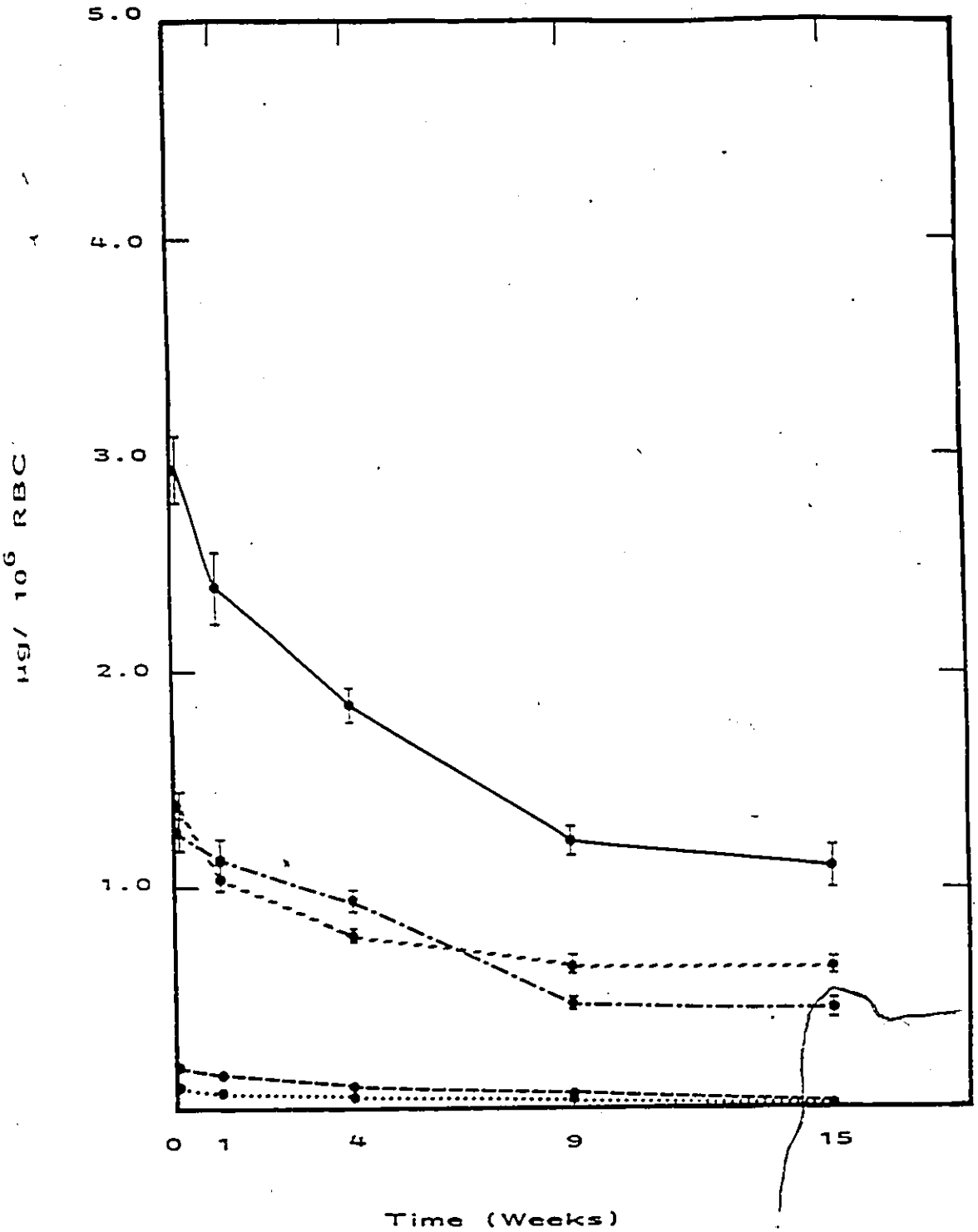
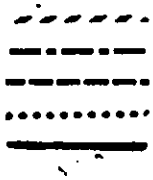


Figure 8: Plot of Males Group 4 (n-6) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex(°) significance at p=0.01 is provided in Tables 6 to 10.



18:2
20:4
22:4
22:5
total (n-6)

Figure 8

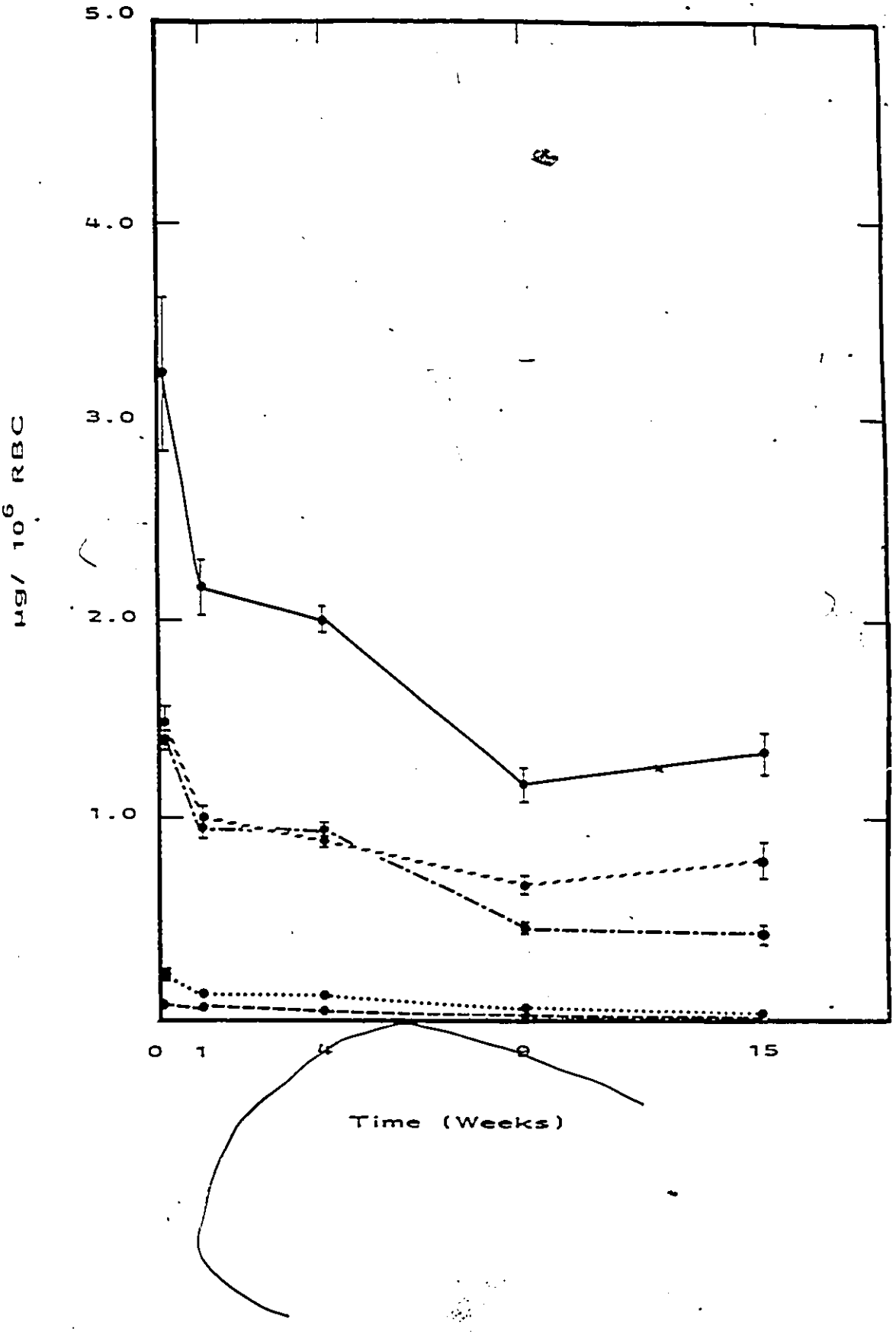


Figure 9: Plot of Females Group 4 (n-6) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 6 to 10.

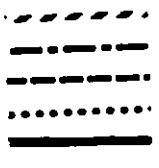
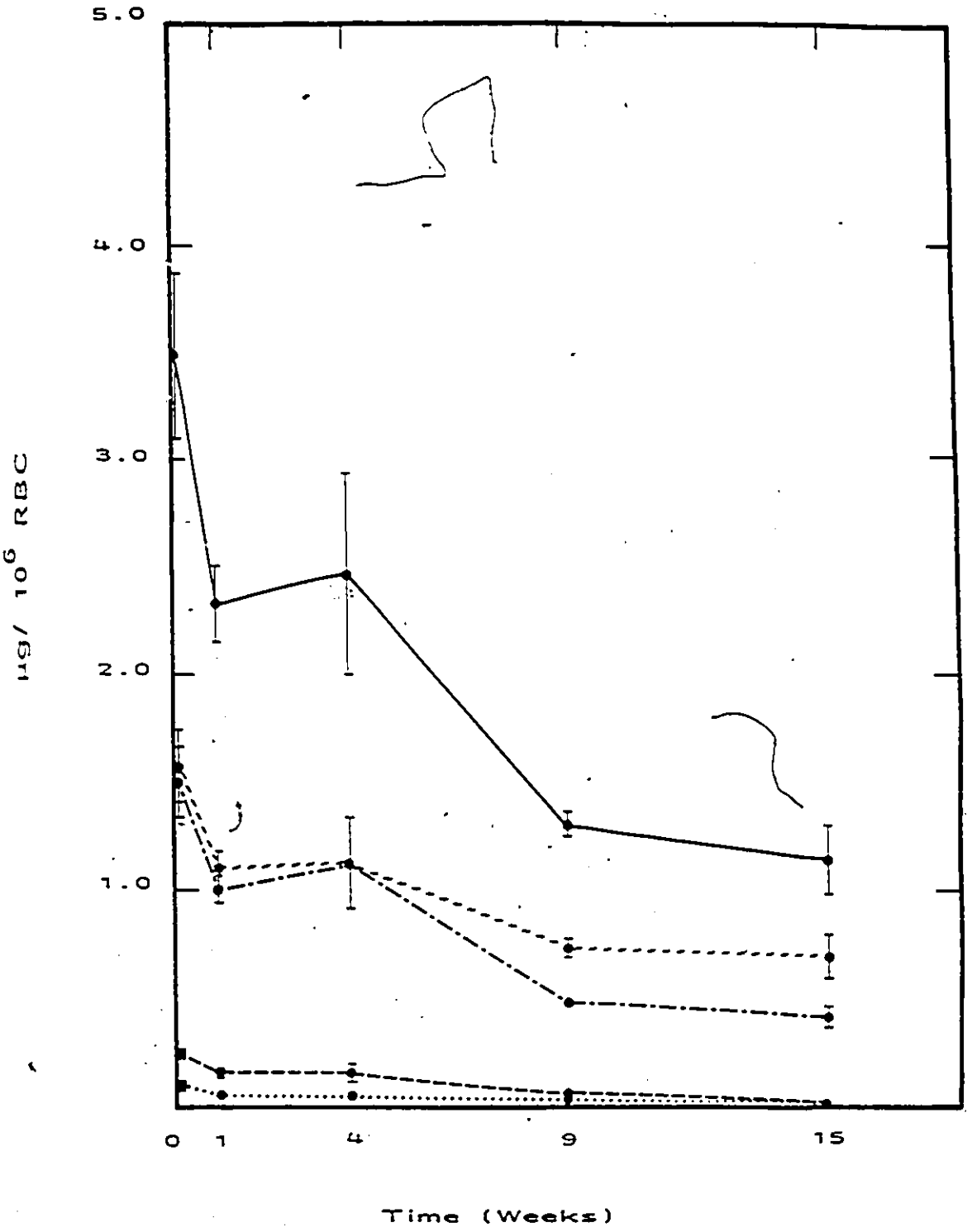


Figure 9



## Erythrocyte Fatty Acid Composition

Figures 10 through 17 represent the (n-3) fatty acyl components of Cynomolgus monkey erythrocytes obtained by gas chromatographic analysis. Diet 1 contained lard and corn oil (high linoleic acid), Diet 2 contained lard and linseed oil (high linolenic acid) and Diets 3 and 4 contained lard, corn oil and menhaden oil (high long chain n-3). All diets were isocaloric and provided equal total polyunsaturate contents; however, diet 1 provided (n-6), diet 2 equal (n-6) to (n-3), diets 3 and 4 equal (n-6) to longer chain (n-3) fatty acids, as the source of polyunsaturates. Diet 4 differed from diet 3 by a four-fold enrichment of alpha-tocopherol.

Figure 10: Plot of Males Group 1 (n-3) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 11 to 16.

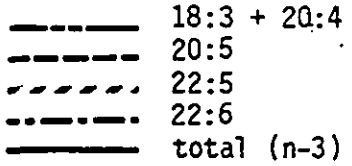


Figure 10

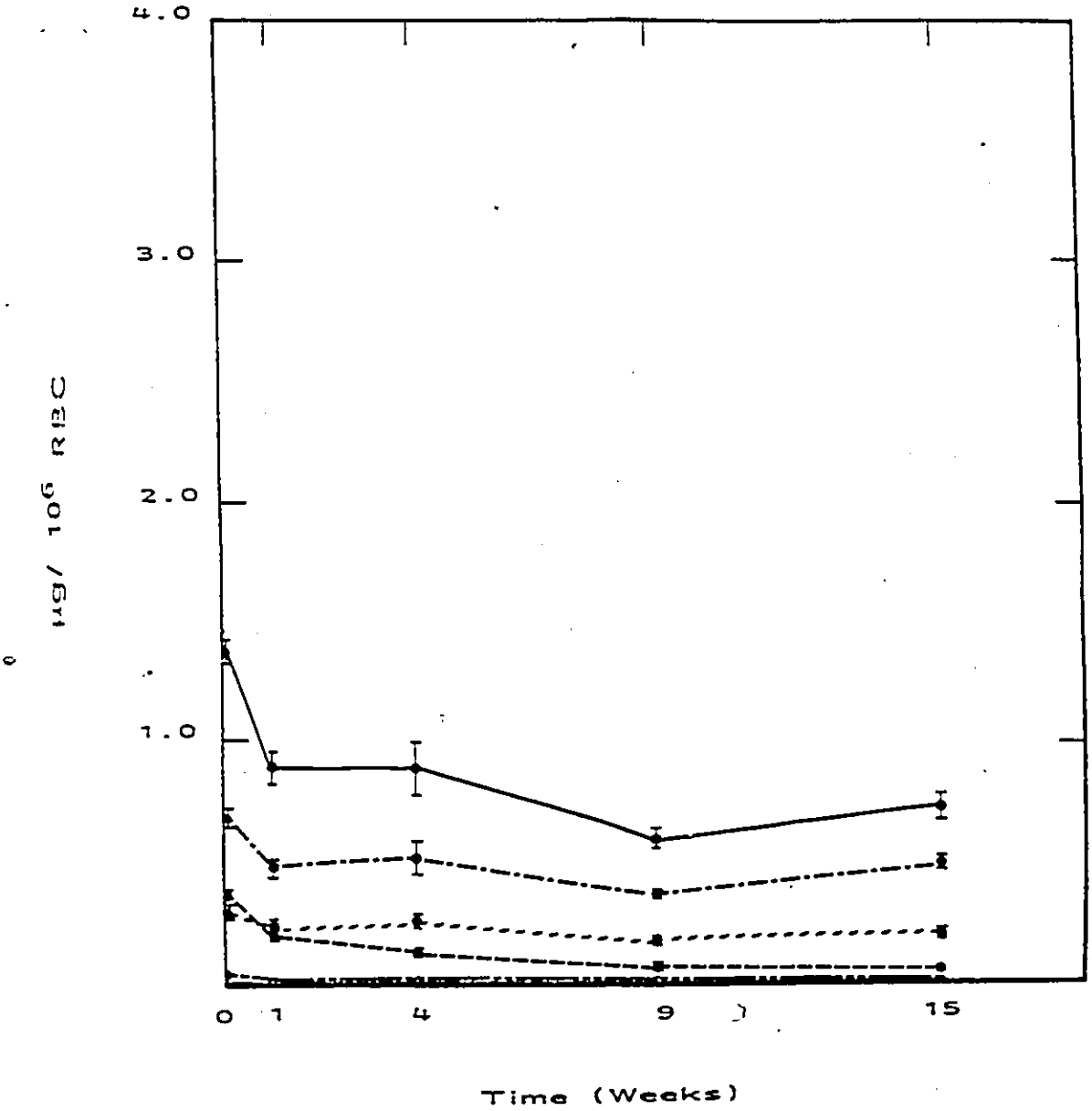


Figure 11: Plot of Females Group 1 (n-3) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 11 to 16.

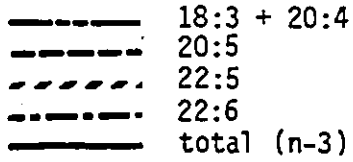


Figure 11

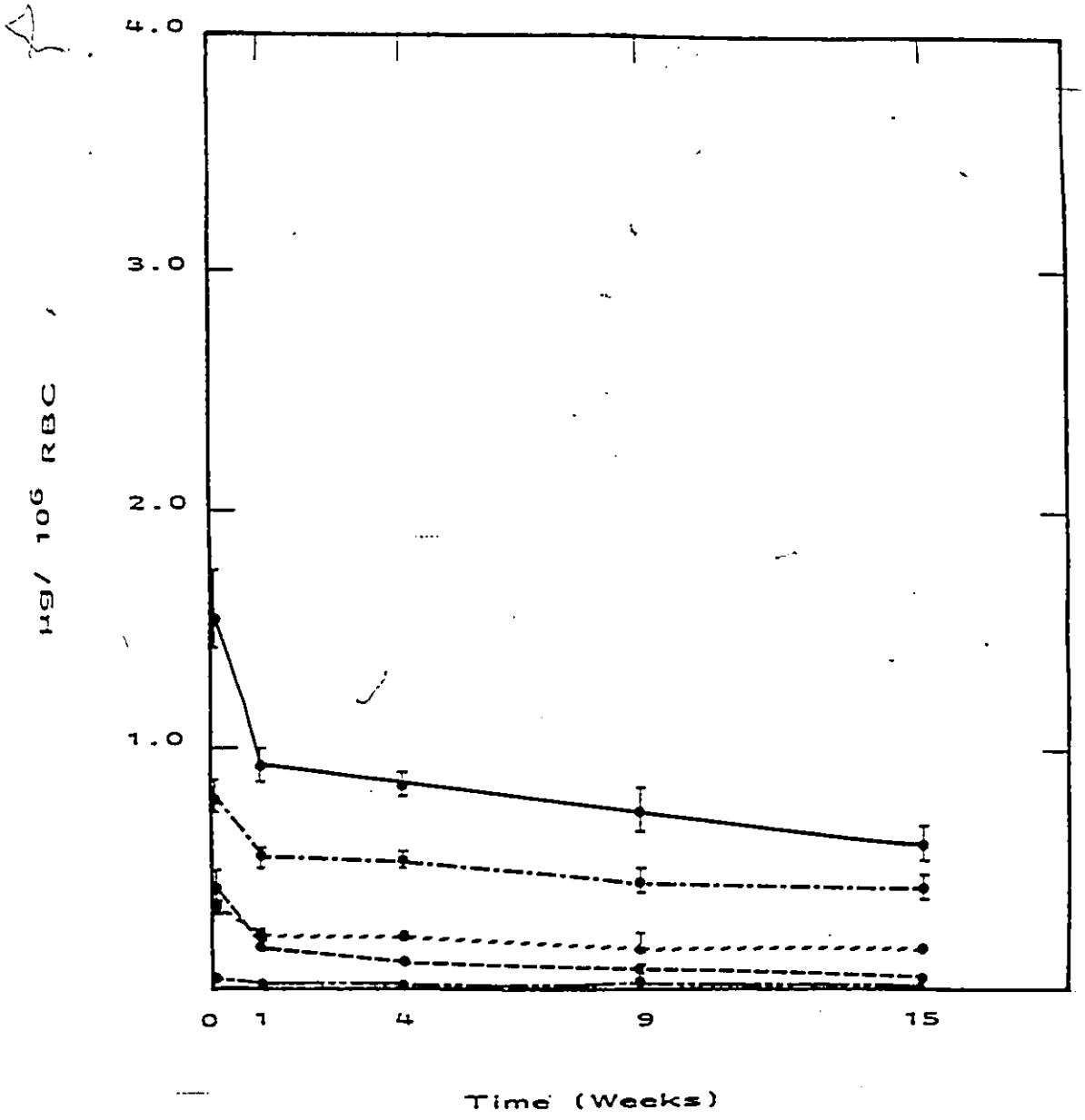


Figure 12: Plot of Males Group 2 (n-3) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 11 to 16.

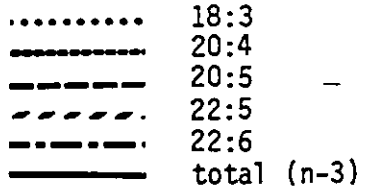


Figure 12

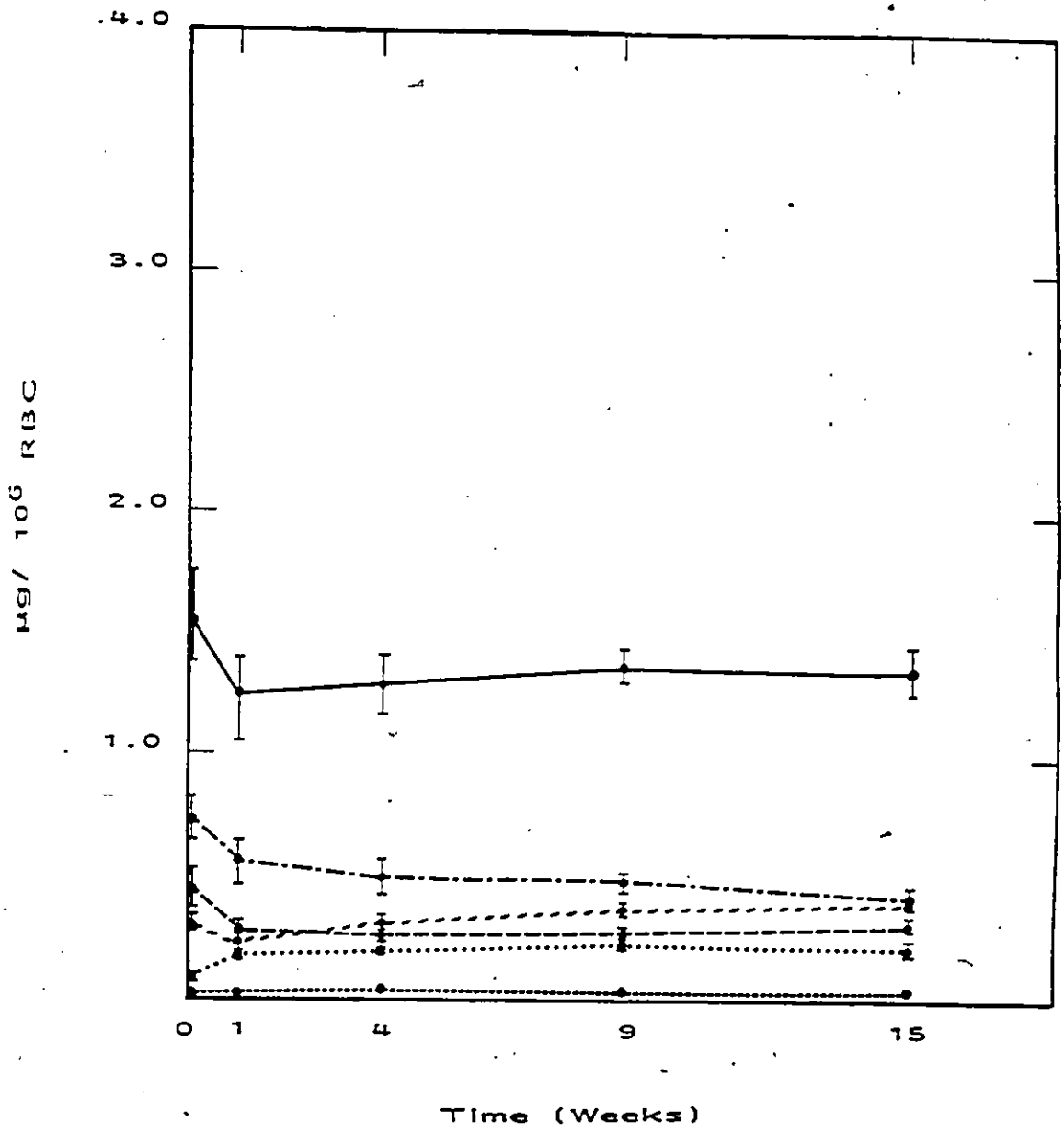


Figure 13: Plot of Females Group 2 (n-3) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significances at p=0.01 is provided in Tables 11 to 16.

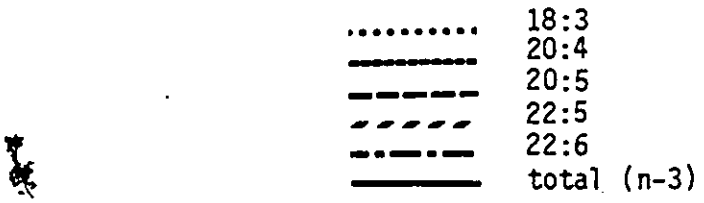


Figure 13

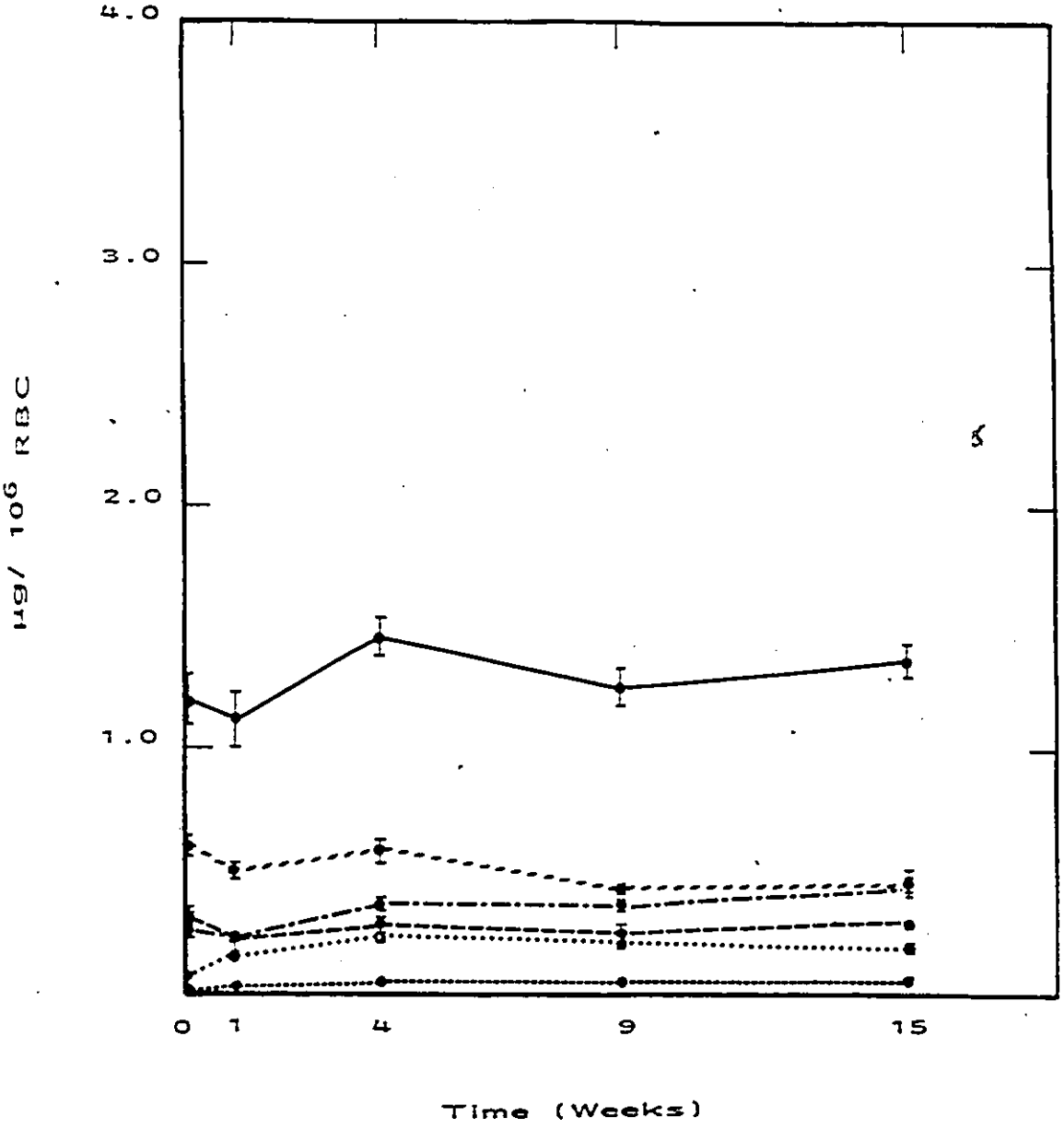


Figure 14: Plot of Males Group 3 (n-3) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (•) significance at p=0.01 is provided in Tables 11 to 16.

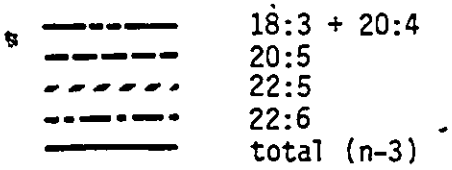


Figure 14

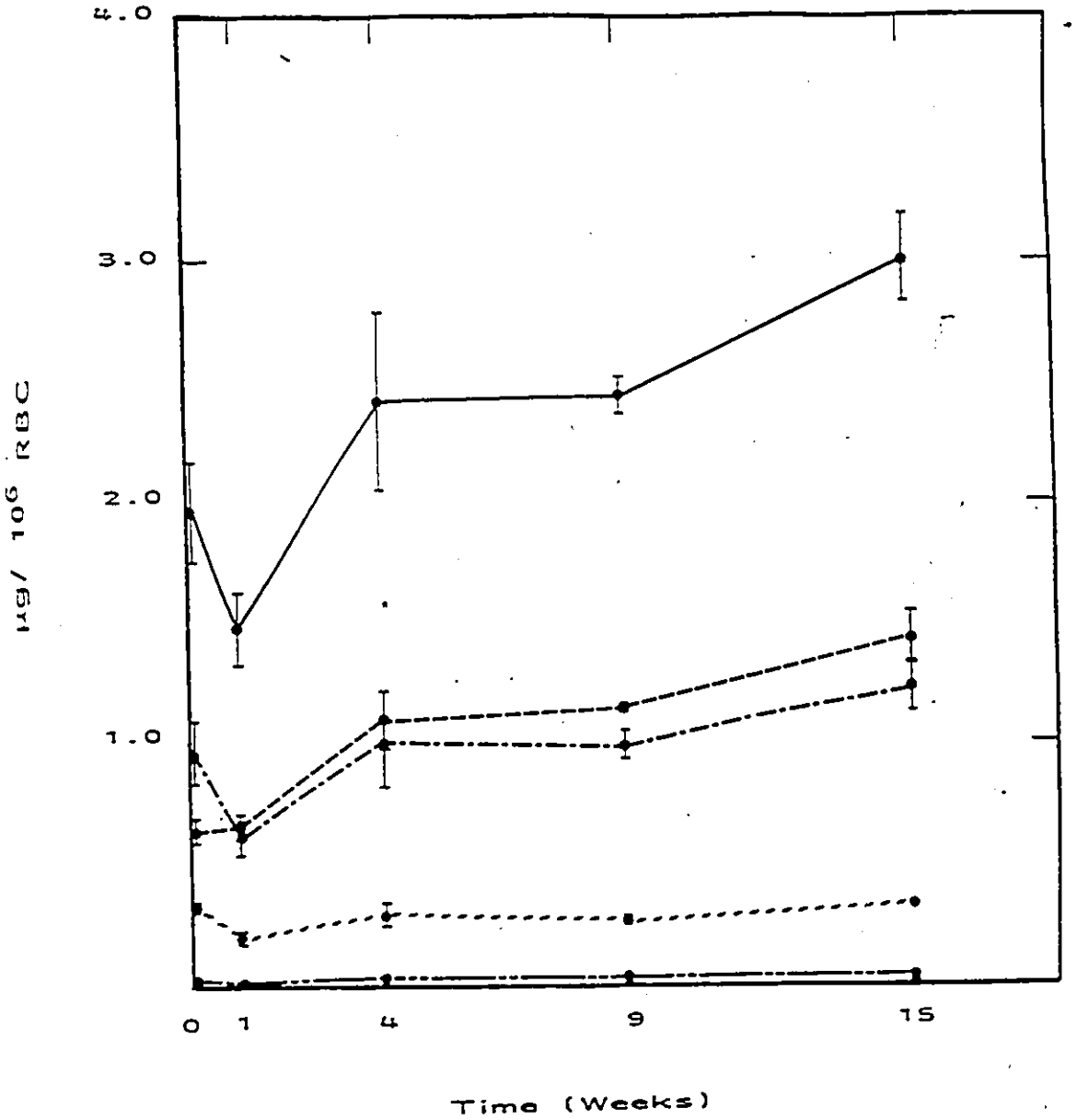


Figure 15: Plot of Females Group 3 (n-3) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 11 to 16.

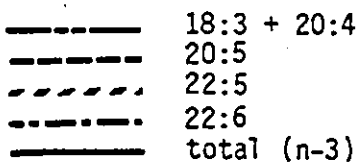


Figure 15

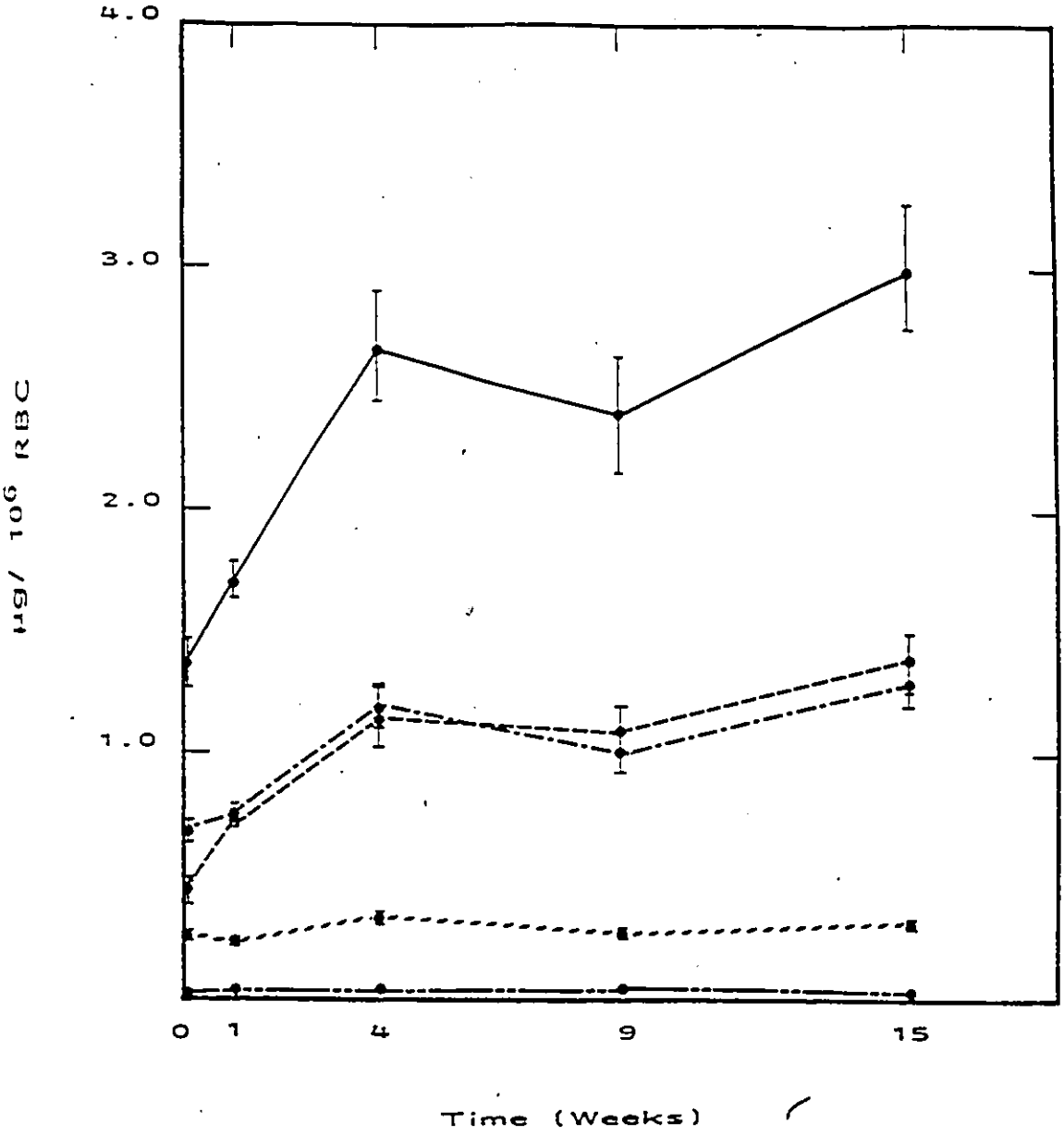


Figure 16: Plot of Males Group 4 (n-3) Fatty Acids ($ug/10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significance at p=0.01 is provided in Tables 11 to 16.

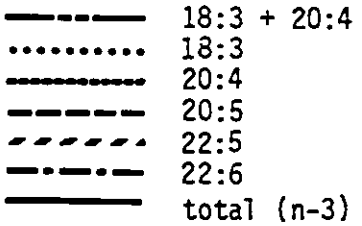


Figure 16

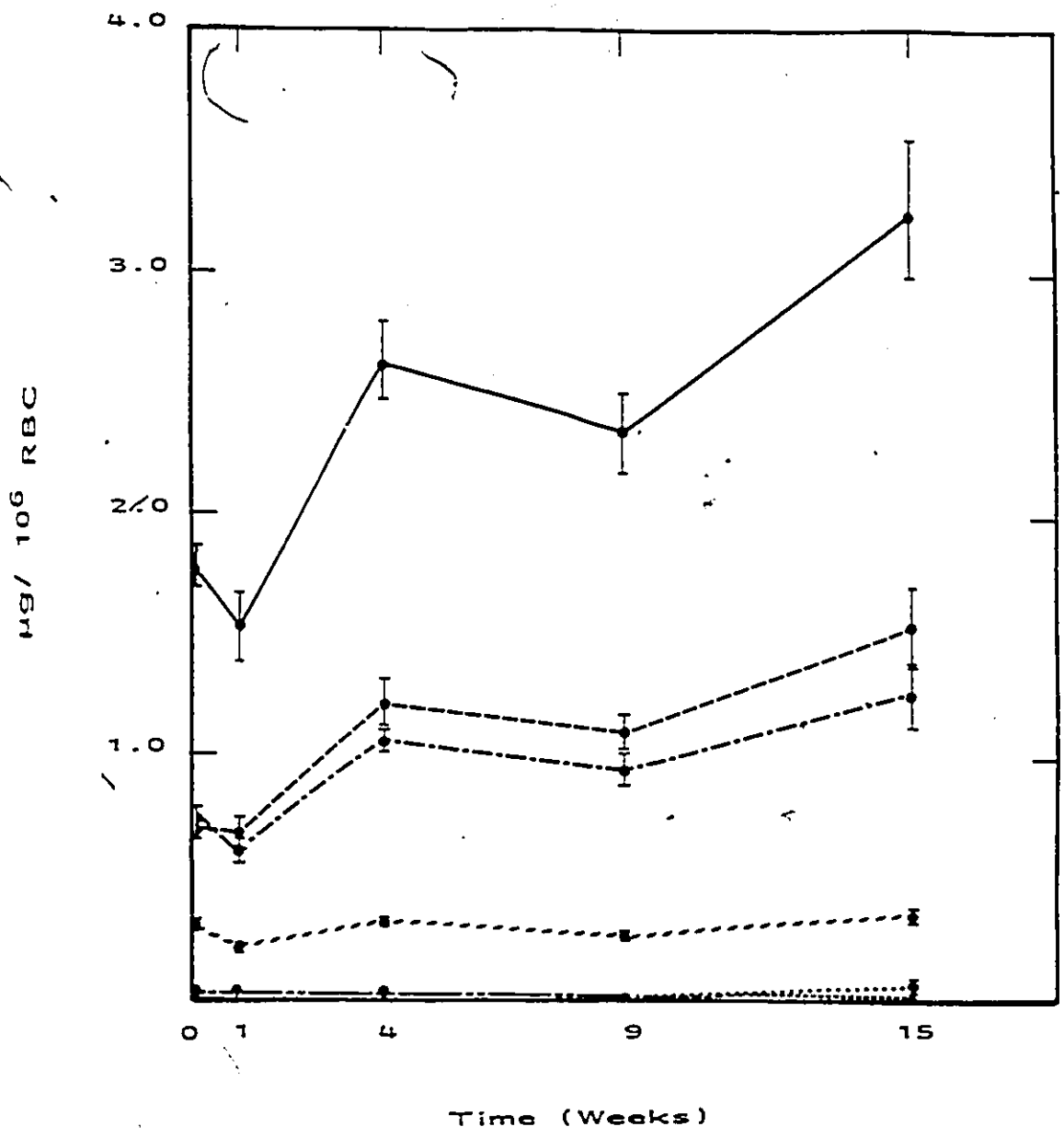
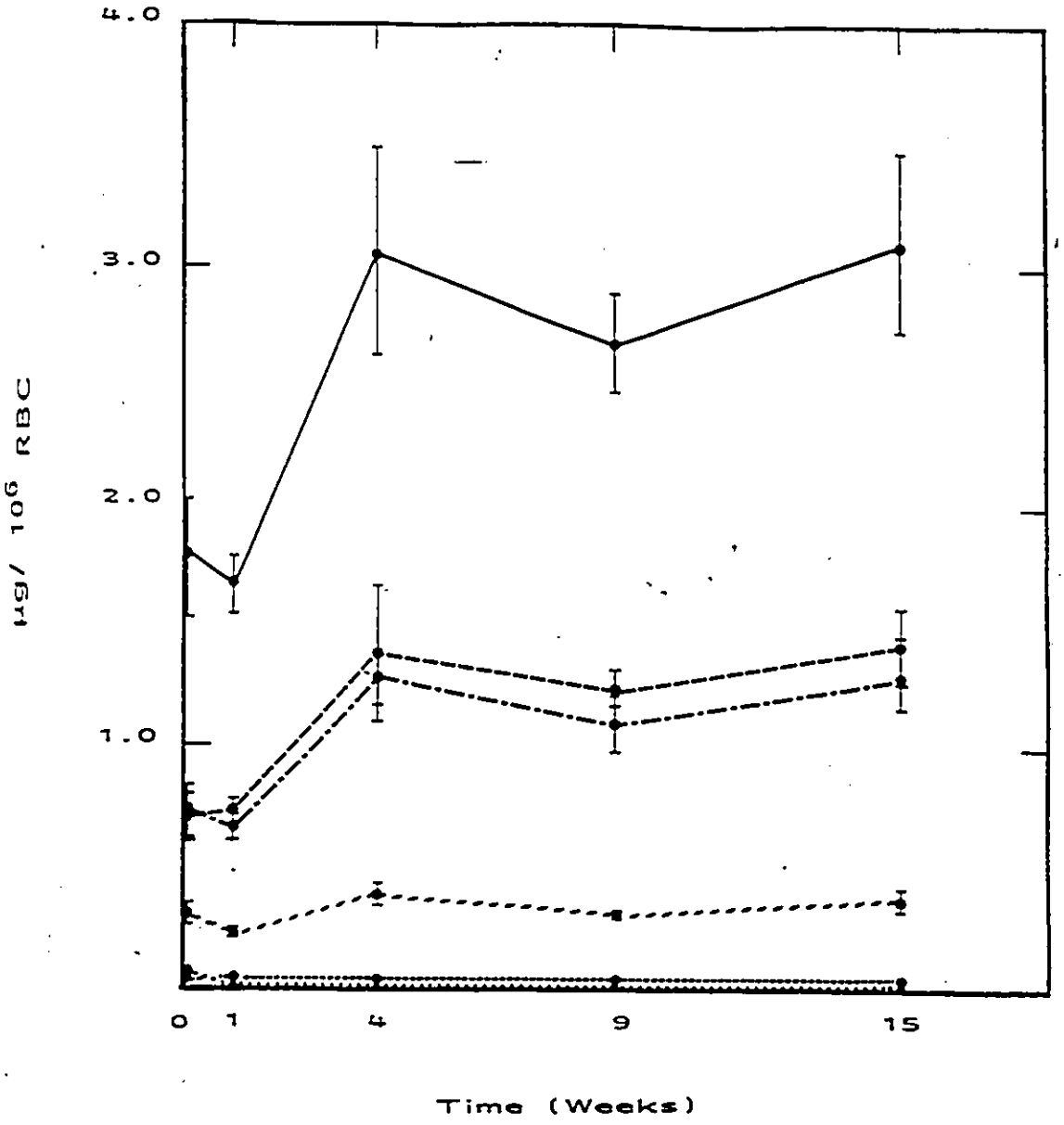


Figure 17: Plot of Females Group 4 (n-3) Fatty Acids (ug/$10^6$ RBC) vs. Time

Values represent group means +/- S.E.M. of fatty acid content at weeks 0, 1, 4, 9 and 15 of study. Post hoc testing of means for group (*), time (†) and sex (°) significances at p=0.01 is provided in Tables 11 to 16.

| | |
|---|---|
| ..... | 18:3 |
| ———— | 20:4 |
| — — — — | 20:5 |
| - - - - - | 22:5 |
| —·—·— | 22:6 |
| ———— | total (n-3) |

Figure 17



# Curriculum Vitae

NAME: MARTHA ANN CARMAN

DATE OF BIRTH: April 15, 1960

PLACE OF BIRTH: Chatham, Ontario

CITIZENSHIP: Canadian

EDUCATION:

Confederation High School
Nepean, Ontario (Grades 9-13)

University of Guelph
Guelph, Ontario
B.Sc. (Hons) Food Science, 1983

University of Ottawa
Ottawa, Ontario
Department of Biochemistry (registered 1983-present)

AWARDS:

Honours Society, 1974-1979

Ontario Scholar, 1979

Robert Arthur Stewart Entrance Bursary
University of Guelph, 1979

Graduated with Honours
University of Guelph, 1983

EXPERIENCE:

Naval Reserve Summer Student Training Program
Royal Canadian Navy
HMCS Carleton, CFB Dow's Lake
May 1979-September 1979, May 1980-September 1980

Student Research Assistant--Radiopharmaceuticals
Career Oriented Summer Employment Program (COSEP)
Radiation Protection Bureau
Health Protection Branch
Health and Welfare Canada
May 1981-September 1981, May 1982-September 1982

Chemist
Bureau of Nutritional Sciences
Health Protection Branch
Health and Welfare Canada
May 1983-October 1986

Analytical Chemist/Flavourist
Griffith Laboratories
Scarborough, Ontario
November 1987-present

Martha Ann Carman

PUBLICATIONS AND PRESENTATIONS:

(n-3) and (n-6) Fatty Acid Status of Canadians
Meeting of American Oil Chemists' Society, Ottawa,
October 1985

Carman, M.A. and Beare-Rogers, J.L. (1987)
Influences of (n-3) and (n-6) Fatty Acids in Monkey
Erythrocytes. Lipids (in press)

COURSES TOWARDS M.Sc.

| | |
|---|---|
| Thesis Research in Biochemistry | BCH 6001-6003 |
| General Biochemistry | BCH 8201 |
| Membrane Biochemistry | BCH 8116 |
| Advanced Topics in Biochemistry | BCH 8130 |
| Advanced Topics in Gene Expression | BCH 8119 |

Seminars

| | |
|---|---|
| The Haemostatic Role of Dietary Eicosapentaenoic Acid in the Prevention of Thrombosis | 1984 |
| Lipid Asymmetry in Human Erythrocytes | 1985 |
| The Lipid Research Clinic Coronary Primary Prevention (LRCCPPT) Trial | 1986 |

| | |
|---|---|
| Introduction to Experimental Biochemistry (BCH 2936) | Laboratory Demonstrator |